Amino Acid Composition of Fishery Products (II)

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ABSTRACT

The essential amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, tryptophane and tyrosine were determined in the following fishery products using microbiological assay technique: fish meals, stickwaters (fish solubles), condensed fish solubles, liver, commercial liver hydrolysate, frozen pink salmon viscera, chum salmon fingerlings and herring scales.

In a previous paper (Deas and Tarr 1949) the essential amino acid composition of certain enzyme- or acid- or alkali-hydrolysed fishery products was given. This work has been extended to include analyses of a number of fish meals, stickwaters and certain other marine products. Brief reports regarding the results have already been made (Deas, Ney and Tarr 1948, 1949).

The fish meals were obtained from two different reduction plants and included those prepared from whole herring, from salmon offal discarded during the canning process or from waste from filleting different varieties of flounders (soles). In certain instances these meals had been prepared by a vacuum drying process, and in others by flame drying. The stickwaters were frozen shortly after collection and stored thus until they were hydrolysed directly for analysis. The procedures for drying and extracting materials with acetone, and for hydrolysing them with acid or alkali for determination of essential amino acids were described in the paper referred to above. The addition of cysteine to certain proteins prior to hydrolysing them with alkali for determination of tryptophane and tyrosine has been found to increase recovery of these amino acids by microbiological assay procedures (Kuiken, Lyman and Hale 1947). In order to test this method with certain fish meals 100 mg. of cysteine was added per 500 mg. of extracted meal before hydrolysing with alkali *in vacuo* in sealed glass tubes.

The essential amino acid composition of the various materials analysed is given in table I. The results show that the fish meals had a consistently high and very similar distribution of these amino acids in spite of the rather diverse nature of the materials used in their preparation. The recovery of tryptophane and tyrosine was not substantially increased in the meals when cysteine was added prior to alkaline hydrolysis. The same uniformity of distribution was observed

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TABLE I. Essential amino acids in acid and alkali hydrolysed fish products (g. of amino acid per 16 g. of nitrogen).

Material hydrolysed	Nitrogen %	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Valine	Tryptophane	Tyrosine
Meals												
Herring	11.3	8.0	2.3	6.6	8.1	8.3	2.9	4.5	5.1	5.8	0.96	1.4
Herring	12.7	7.9	2.4	6.7	9.4	8.0	2.8	4.4	5.1	5.9	0.88	1.4
Herring	13.2	8.3	2.6	6.8	9.3	7.9	2.7	4.3	5.5	5.9	1.2	1.6
*Pink salmon and "soles" (A)	11.2	8.1	2.9	5.5	9.5	11.2	2.4	4.8	5.2	5.5	1.0(0.79)**	2.8(3.4)
*Chum salmon (B)	11.1	8.4	2.8	5.9	10.8	12.2	2.6	5.3	5.4	6.8	1.0(0.84)	3.0(2.9)
*Pink salmon (C)	11.6	7.1	2.4	5.1	8.5	10.8	2.4	4.5	5.0	4.8	0.88(0 76)	2.6(2.8)
*Pink salmon and "soles" (D)	10.9	7.1	2.6	5.5	8.5	12.5	2.5	4.4	5.1	5.7	0.91(0.75)	2.9(2.8)
*Pink and sockeye salmon (E)	10.7	8.6	2.5	4.5	9.1	11.0	2.6	4.4	5.1	5.5	0.89(0.69)	2.8(2.9)
*Pink salmon (F)	10.7	9.0	2.6	6.4	9.7	12.7	2.8	4.5	5.6	8.0	0.95(0.84)	3.0(3.2)
Sockeye salmon	9.8	8.3	2.5	5.6	7.8	10.4	2.6	3.4	4.6	6.3	0.89(0.83)	3.1(3.6)
Stickwaters												
Herring	0.84	5.9	1.1	2.3	4.2	5.3	1.9	1.8	2.6	2.4		0.3
Herring	0.90	5.8	1.6	2.0	4.1	7.1	1.6	2.6	2.5	2.8	0.24	0.41
Pink salmon and "soles" (A)	0.61	8.1	1.7	2.2	3.7	7.7	2.0	2.1	2.9	2.1	0.26	0.64
Chum salmon (B)	0.60	7.8	1.7	2.2	3.7	8.1	1.9	2.1	2.9	2.7	0.24	0.57
Pink salmon (C)	0.77	8.2	1.6	2.4	3.5	7.3	2.0	2.1	2.8	2.6	0.26	0.6
Pink salmon and "soles" (D)	0.72	7.9	1.6	2.6	4.0	6.3	1.8	2.3	2.9	2.9	0.32	0.64
Pink and sockeye salmon (E)	0.68	7.7	1.6	2.3	3.3	6.8	2.0	2.2	2.9	2.5	0.28	0.68
Pink salmon (F)	0.59	7.8	1.6	1.5	3.5	6.6	2.1	2.0	3.4	2.5	0.38	0.79

TABLE I. (*cont'd*) Essential amino acids in acid and alkali hydrolysed fish products (g. of amino acid per 16 g. of nitrogen).

Material hydrolysed	Nitrogen %	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Valine	Tryptophane	Tyrosine
Condensed fish solubles												
Sample 1	4.4	4.5	1.0	4.5	6.2	4.1	2.6	3.0	2.2	3.9	–	1.4
" 2	6.2	1.4	0.5	1.8	2.4	3.2	0.7	1.1	1.2	1.9	1.2	0.29
" 3	4.3	3.2	1.8	2.2	3.4	5.0	1.5	1.7	2.1	2.0	2.4	0.35
" 4	5.1	4.7	–	2.5	4.4	5.7	1.8	2.0	2.4	2.7	2.0	0.40
Liver												
From red cod	11.3	7.2	2.7	5.5	8.4	8.0	3.0	5.8	4.8	5.8	1.2	1.5
†Commercial hydrolysate	2.5	0.12	0.86	4.0	5.0	0.27	2.1	3.2	2.9	4.5	0.046	0.31
†Commercial hydrolysate after acid or alkali hydrolysis	2.2	0.17	0.87	4.0	5.3	0.8	2.0	3.3	3.1	4.7	0.10	0.50
Miscellaneous												
Chum salmon fingerlings (3 months old)	13.0	6.8	3.1	9.8	10.7	7.7	3.0	5.7	5.7	–	0.76	1.5
Chum salmon fingerlings (3½ months old)	13.2	6.8	3.1	9.4	10.6	7.6	3.0	5.6	5.7	6.4	0.90	1.9
Pink salmon viscera (frozen sample)	14.7	7.3	3.1	8.4	10.7	7.9	3.3	5.1	5.2	6.6	1.1	1.9
Herring scales	11.8	7.6	1.9	1.9	4.2	5.1	2.8	2.7	3.3	3.0	0.1	0.55

*Meals prepared by vacuum drying, remainder by flame drying.
†Prepared from Atlantic cod livers: analysed before and after further hydrolysis with HCl or NaOH in the usual manner.
**Figures in parenthesis are results obtained with meals which were hydrolysed by alkali in vacuo in the presence of cysteine.

in stickwater samples, but in general the concentration of amino acids was considerably lower. The four samples of condensed fish solubles examined contained variable amounts of essential amino acids. Two of them, particularly sample 2, possessed a rather foul odour indicative of considerable bacterial putrefaction. Recent work by Lassen, Bacon and Dunn (1949) has shown that such putrefaction may result in a marked destruction of essential amino acids, and this may well have accounted for the irregular results obtained. The high tryptophane results for condensed fish solubles are difficult to explain. It is possible that products of bacterial putrefaction stimulated response of the test organism in the microbiological assay for tryptophane.

Analyses of the chum salmon fingerlings showed that they contained over four times as much lysine, more than twice as much histidine and somewhat more arginine than whole eggs (roe) of salmon or herring (Deas and Tarr 1949). The essential amino acid distribution in these fingerlings was very similar to that of the sample of pink salmon viscera and did not vary greatly from that of fish flesh previously analysed (Deas and Tarr 1949).

The essential amino acid distribution in the sample of fish liver was closely similar to that found in fish meals and did not vary greatly from that of mammalian liver (Block and Bolling 1945, p. 303). The liver hydrolysate was markedly deficient in the three basic amino acids, arginine, histidine and lysine. Since amino acid recovery was not affected by further hydrolysis of this material it is assumed that hydrolysis must have been fairly complete in the original solution.

Analysis of herring scales indicated an almost complete absence of tryptophane.

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The Influence of Trimethylamine Oxide on the Bacterial Reduction of Redox Indicators

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ABSTRACT

In the presence of bacteria capable of reducing it, trimethylamine oxide exerts a poising action on the oxidation-reduction potentials of media. This poising is at an Eh level considerably electropositive to the Eó of the redox indicators commonly used in the "reduction tests" used for determining the bacterial quality of foods.

At various times, attempts have been made to use the methylene blue reduction test as a measure of bacterial contamination on fresh fish. The results have not been satisfactory. When applied to suspensions of fish muscle, in a manner similar to that used with milk, the reduction of the dye is neither fast enough, sensitive enough nor reliable enough to be of any practical use.

The obvious question is: Why? Why do bacteria apparently have greater difficulty in reducing a redox indicator in a suspension of fish muscle than they do in a sample of market milk?

The reduction of methylene blue, or the lowering of the oxidation-reduction potential in these foods is brought about by several different mechanisms. Chief among these are the removal of dissolved oxygen, either mechanically or by the respiration of living cells; the reducing activity of various substances in the substrate; the release of reducing agents from the substrate through microbial decomposition (hydrogen, hydrogen sulphide, etc.) and the enzymatic activation of sluggish or inert oxidation-reduction systems.

Thus, it can be seen that the nature of the substrate as well as the activity of the micro-organisms determines the rate at which the oxidation-reduction potential is lowered. A typical illustration of this is seen in the recent work of Hirschmann and Lightbody (1947). They found that changes occurring in egg powders during drying and storage altered the relationship between the size of bacterial populations and the rate of dye reduction.

It is obvious that the use of redox indicators as a measure of the bacterial population in a food will be valid only when the oxidation-reduction potential, or changes in the potential are predominantly the result of bacterial activity and not as a result of the poising or reducing action of the food itself.

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The muscle tissues of cod, haddock, halibut, hake and many other marine fish are characterized by the presence of relatively large amounts of trimethylamine oxide (Beatty 1939). Through the agency of microbial enzymes this is readily converted into trimethylamine (Watson 1939, Tarr 1939). It would appear that this system, $CH_3N \rightleftarrows CH_3NO$, belongs to group III in the classification suggested by Barron (1939), which consists of electrometrically sluggish oxidation-reduction systems requiring the mediation of a specific enzyme. Tarr (1940) has shown the specificity of this particular enzyme.

There is little or no indication in the literature of the position on the Eh scale where the trimethylamine oxide—trimethylamine system belongs. Because it is so characteristic of these marine fish, and always present in significant amounts, it seemed worthwhile to ascertain whether it could have a significant poising action on the oxidation-reduction potentials of fish, and indirectly affect the reduction of methylene blue.

To do this, several different experiments were made: (1) Using Washed cells of an oxide-reducing culture in Thunberg tubes, the poising action of the oxide was determined by a series of redox indicators. (2) Somewhat similar tests were made by growing oxide-reducing bacteria in broth cultures containing these same redox indicators. (3) Eh-time curves were plotted from potentiometric measurements made on culture media, with and without trimethylamine oxide and inoculated with pure cultures of bacteria.

EXPERIMENTAL PROCEDURES

The nutrient broth used in these experiments consisted of the following ingredients and percentages: glucose 0.25, Bacto-peptone 0.5, sodium chloride 0.5, magnesium sulphate 0.1, dipotassium phosphate 0.1. Unless otherwise mentioned the pH was approximately 7.2. All the redox indicators, except resazurin, were B.D.H. products. The resazurin was obtained from Anachemia Limited.

POTENTIOMETRIC METHODS

The measurements of oxidation-reduction potentials were made in the conventional manner using platinum electrodes, saturated potassium chloride agar bridges, a calomel half cell and a Cambridge potentiometer.

The electrodes used in these tests were selected by checking them in a standard solution. Those deviating more than 0.02 volts from the norm in a group of 8 or more were discarded. The electrodes were cleaned by successive treatments with "Bon Ami", benzene, chromic acid cleaning solution and dilute hydrochloric acid. Between each treatment they were thoroughly rinsed in distilled water. In some of the later tests the electrodes were cleaned by rinsing in distilled water and then heating the platinum wire to white heat in a bunsen burner.

It was occasionally observed that electrodes, remaining continuously in a heavy suspension of bacteria, registered potentials different from those given by

freshly cleaned electrodes recently inserted into the same cell suspension. For example, the potential in a heavily seeded culture showed an initial drop in the first six hours to a value of − 0.11 volts. At 24 hours this rose to + 0.04 volts and after 96 hours it was + 0.076 volts. At this point one of the electrodes was carefully removed, cleaned and re-inserted. After a short time the potential came to equilibrium at − 0.099 volts, which is a close approximation to the low range reached after the first six hours. In such instances, the lower reading given by the recently cleaned electrode was always accepted as the true value.

EXPERIMENTAL RESULTS

A. Observations with Indicators

1. Using the Thunberg technique, tests were made to determine the ability of mass inoculations of washed cells of *Ps. putrifaciens* to reduce a series of redox indicators in the presence and absence of trimethylamine oxide. Suspensions of these oxide-reducing bacteria were prepared in buffer solution pH 7, and sodium acetate was added to act as a hydrogen donor. It will be seen, table I, that only

Table I. Reduction of redox indicators by mass inoculation of "washed" trimethylamine oxide reducing bacteria in the presence and absence of trimethylamine oxide, using the Thunberg technique with the pH buffered at 7.0.

Indicator	Eó at pH 7.0	Reduction of Indicator*	
		Oxide Present	Oxide Absent
O-Bromophenol-indophenol	+.230	++	++
Bindschedler's green	+.224	++	++
O-cresol indophenol	+.191	++	++
Thymol indophenol	+.174	++	++
1. Naphthol-2-sodium sulphonate-indo-2:6-dibromophenol	+.119	+	+
Tolylene blue	+.115	+	+
Thionine	+.063	−	+
Brilliant cresyl blue	+.047	−	+
Methylene blue	+.011	−	+
Potassium indigo-trisulphonate	−.081	−	+
Nile blue	−.122	−	−
Potassium indigo-disulphonate	−.125	−	−
Janus green	−.255	−	−
Neutral red	−.325	−	−

*++ rapid or almost instantaneous reduction
+ reduction within 15 minutes
− not reduced after 2 hours incubation

those dyes whose Eó at a pH of 7.0 is + .115 volts and above were reduced in the tubes containing the oxide, while the dyes in the tubes, without the oxide having an Eó of − .081 and above were reduced. This would indicate that the poising

action of trimethylamine oxide commences at a level somewhere between + .063 and + .115 volts.

2. A nutrient broth, to which was added resazurin, (E6 at pH 7.0 approximately − .05 volts, Twigg 1945) to give a concentration of 1:200,000 was divided into 2 parts and trimethylamine oxide was added to one portion to give a concentration of 0.5 per cent. These were inoculated in duplicate with equal quantities of a suspension of *Ps. putrifaciens*, and sets were incubated at 25°C, 7°C and 3°C. In every instance the dye was reduced in the tubes containing no oxide and not reduced up to 20 days in the tubes containing the oxide.

To ensure that this result was not caused by inhibition of bacterial growth in the presence of the oxide, bacterial plate counts were made periodically. It was found, for example, that in the cultures incubated at 7°C, shortly after the dye had been reduced in the tubes without the oxide the counts from duplicate tubes were 23×10^6 and 31×10^6, per ml; while those in the tubes containing oxide were 37×10^6 and 45×10^6, but the indicator remained oxidized. The counts at the other temperatures gave precisely the same picture; in spite of very large numbers of bacteria the oxide poised the potential above the Eh where resazurin is reduced.

Similar inoculations were made into these resazurin broths from 38 pure cultures, and incubated at 25°C. Four different reactions were observed.

A. Certain of the marine *micrococci* and *Flavobacteria* grew slowly but did not reduce the indicator in either set of tubes.

B. *Bacillus subtilis*, *B. mycoides*, *Mic. citreus*, *Mic. candicans* and seven green *Pseudomonas* cultures reduced the indicator in the tubes with and without the oxide; in some instances the reduction of the dye in the tubes containing the oxide was slightly slower, but once reduced it remained reduced for several days.

C. *Esch. coli*, and some strains of *Aer. aerogenes*, *Prot. vulgaris*, *Sar. marcescens* and *Ach. candicans* reduced the dye only in the tubes without the oxide.

D. Certain strains of the last four species rapidly reduced the dye in both sets of tubes, but shortly afterwards the oxide tubes became reoxidized and remained so throughout the remainder of the incubation period.

These results indicate that oxide definitely poised the potential of the media above the level where resazurin was reduced only in the case where the bacteria are able to reduce the oxide (group C and D). It is interesting to observe that in some of these cultures the bacteria were able to bring about a rapid reduction of the potential in the presence of the oxide for a short period—perhaps before sufficient enzyme was formed to act on the oxide. But this initial reduction was only temporary, and as indicated by the reoxidation of the dyes, soon rose again to a potential above that of the oxidized thionine.

3. A nutrient broth was made up with and without 5 per cent trimethylamine oxide. The redox indicators used in the previous tests were added and the broth autoclaved in small screw-capped bottles. These were inoculated in duplicate from a suspension of *Ps. putrefaciens* and incubated at 25°C. The results are given in table II. As will be seen by a comparison of tables I and II the results obtained with the large mass of washed cells were somewhat similar but not identi-

cal with the active growing cultures. The active cultures reached a slightly lower potential in the absence of oxide; and the brilliant cresyl blue was reduced in the presence of the oxide by the growing cultures but not by the washed cells.

TABLE II. Reduction of Redox indicators by actively growing cultures of *Ps. putrifaciens* in nutrient broth, with and without the addition of 5 per cent trimethylamine oxide after 48 hours at 25°C.

Indicator	Eo at pH 7.0	Reduction of Indicator*	
		Oxide Present	Oxide Absent
O-Bromophenol-indophenol	+.230	+	+
Bindschedler's green	+.224	+	+
O-cresol indophenol	+.191	+	+
Thymol indophenol	+.174	+	+
1. Naphthol-2-sodium sulphonate-indo-2:6-dibromophenol	+.119	+	+
Tolylene blue	+.115	+	+
Thionine	+.063	+	+
Brilliant cresyl blue	+.047	−	+
Methylene blue	+.011	−	+
Potassium indigo-trisulphonate	−.081	−	+
Nile blue	−.122	−	+
Potassium indigo-disulphonate	−.125	−	+
Neutral red	−.325	−	−

*+ = indicator reduced
− = indicator not reduced

POTENTIOMETRIC TESTS IN ABSENCE OF ENZYMES

The addition of the oxide to various solutions and culture media, in the absence of bacterial enzymes, never gave a consistent poising action to the oxidation-reduction potential as measured by a platinum electrode. Nor did the use of a vacuum make any significant difference. It was noted, however, that although the oxide did not poise the potential at any one level, the solutions containing it gave a higher reading than similar solutions without the oxide. This could best be shown by purposely poising the potential of the solution in a negative reducing range. Incidentally, it was also noted that when certain organic reducing agents, such as sodium thioglycolate, were added to a peptone-methylene blue solution and boiled briefly, the dye was reduced; if trimethylamine oxide was added to the same solution the dye was not reduced.

It would seem that although the oxide-amine system does not establish a potential in the absence of its specific enzyme, the oxide does interfere in some manner with the activity of certain reducing substances which normally affect the potential.

POTENTIOMETRIC MEASUREMENTS IN THE PRESENCE OF BACTERIA

Potentiometric measurements confirmed the results obtained with the indicators. Invariably the presence of the oxide in media inoculated with oxide-reducing bacteria retarded the fall in the potential. The poising action at a definite level could be most easily demonstrated either by adding an excess of oxide or by using cultures with relatively weak reducing powers, such as some of the marine *Micrococci* (figs. 1 and 2).

As might be expected, continued poising at one level was not obtained with vigorous oxide reducers, such as E. coli and other members of the Enterobac-

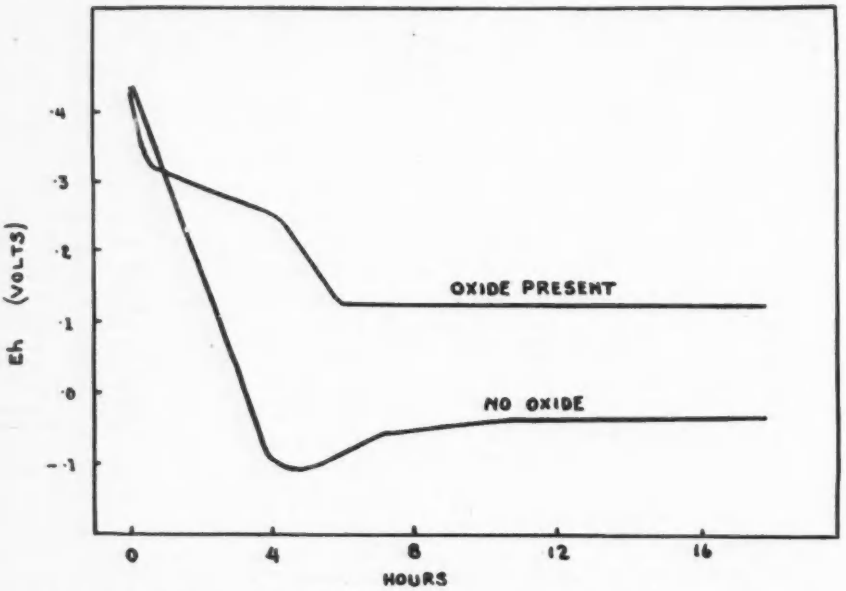

FIGURE 1. Eh-time curves for buffered (pH 7.0) dextrose solution, with and without trimethylamine oxide, and inoculated with a very heavy suspension of *Serratia marcescens*, which reduces the oxide.

teriaceae. Many of these organisms have very strong reducing systems and in the absence of the oxide the potential rapidly dropped to − .2 to − .3 volts or lower. With 1 per cent oxide present to start with there was a much more gradual drop in the potential for the first 12 to 18 hours, followed by a more precipitous incline when the oxide had been almost completely reduced.

It was occasionally observed that potentiometric readings from the tubes containing oxide were much less stable than those obtained from the corresponding tubes without oxide. In several instances it was found that the presence of a redox indicator (resazurin) in the broth corrected this condition and gave a well poised, steadier potential.

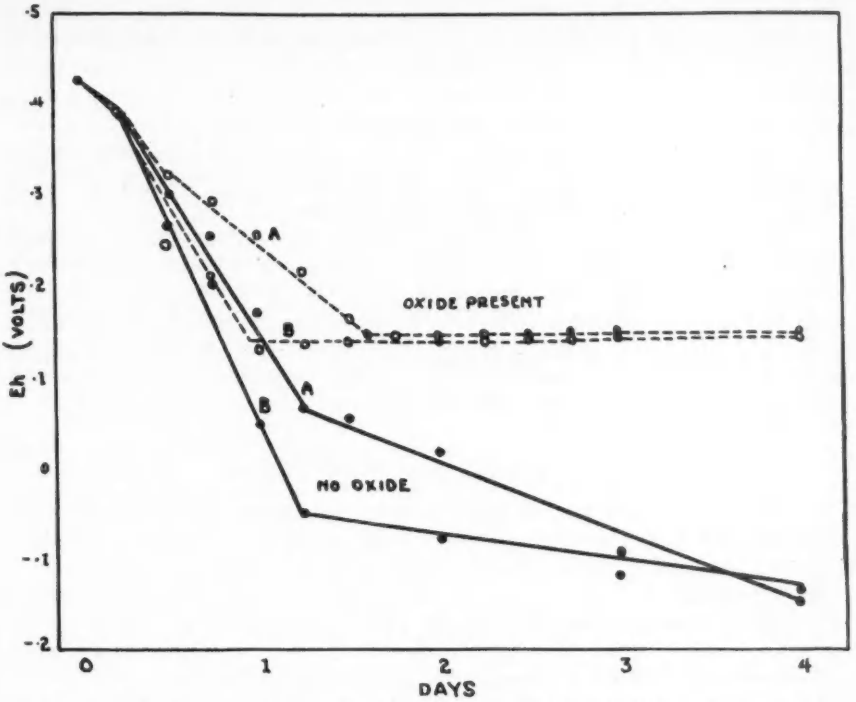

FIGURE 2. Eh-time curves for nutrient broth cultures, with and without 1 per cent trimethylamine oxide and inoculated with two strains of marine *Micrococci*, A and B, which reduce the oxid .

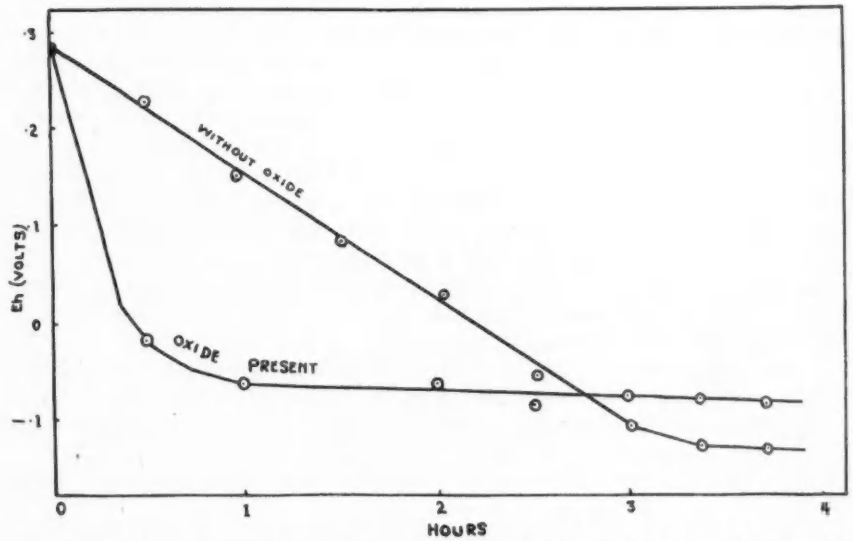

FIGURE 3. Eh-time curves for a nutrient broth with and without added trimethylamine oxide and inoculated with a green *Pseudomonas* of marine origin, which does not reduce the oxide.

When oxide-reducing bacteria were replaced by non oxide-reducing species, the results were different. In some cases it was obvious from the clearness of the broth that the oxide retarded the growth of the cultures, and the apparent poising of the potential was only a reflection of the absence of bacterial activity. Furthermore the level at which this apparent poising occurred was approximately the same as the sterile oxide broth. Such results were obtained with *B. subtilis*, and some of the cultures of *Micrococci* and *Flavobacteria*.

With certain other non-oxide-reducing bacteria, and especially members of the green fluorescent group, the addition of oxide had very little retarding effect on the fall of the potential. In some instances (fig. 3) it was found that the potential actually dropped faster in the presence of the oxide. These results agree with the previous observations (Castell 1946) that the presence of trimethylamine oxide in a culture medium retards the growth of some organisms and has either no effect or a stimulating action on others.

SUMMARY AND DISCUSSION

These observations may be summed up by saying that trimethylamine oxide definitely does have a poising or retarding effect on the lowering of the oxidation-reducing potential in media inoculated with oxide-reducing bacteria.

Although no attempt was made to establish the potentials of the trimethylamine oxide-amine system accurately the results with the indicators show that they are positive to the thionine-reduced thionine system, which has an E_6 of + .063 volts at a pH of 7.0. Potentiometric readings in substrates heavily poised with the oxide show the potential to be in the neighborhood of + .120 to + .140 volts.

This shows that the oxide-amine system is considerably positive to either methylene blue or resazurin, the two indicators that are most frequently used in the so-called "reduction test" for estimating the bacterial content of various foods. It is not surprising therefore, that experience has shown these dyes to be of little use in estimating the bacterial content of marine fish. Most of these sea fish contain from 0.1 to 1 per cent trimethylamine oxide, and the oxide-reducing bacteria are wide-spread among the organisms that contaminate fish.

It is not suggested that the presence of trimethylamine oxide is the only factor which retards the bacterial reduction of methylene blue in suspensions of fish muscle. But it at least gives a partial explanation of the facts already observed; and it suggests further that if the dye reduction tests are to be made more sensitive to bacterial activity, the poising action of the constituents of the muscle must be overcome, or an indicator much more electropositive than methylene blue must be used.

The results obtained with the redox indicators also suggest another relatively simple method for identifying trimethylamine oxide-reducing bacteria. This is based on the fact that the addition of oxide greatly retards the reduction of an indicator, if the organisms in question produce the activating enzyme. A comparison of the reduction time for a dye in nutrient broth with and without oxide

gives the answer. Further details of the technique for this proposed test are now being worked out using resazurin, and the results so far are very encouraging.

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Note on the Colorimetric Estimation of Trimethylamine

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ABSTRACT

Using the Dyer picrate method for trimethylamine determination the Evelyn 400 mμ filter is more satisfactory than the 420 filter.

A few modifications leading to more satisfactory results have been made in the colorimetric method for the determination of trimethylamine as the picrate salt (Dyer 1945) since its publication.

Most difficulties were traced to poor technique in preventing contamination and to wet or dirty colorimeter tubes and apparatus, a fault common in the use of many colorimetric micromethods. The use of stopcock or other grease for lubricating stopcocks on the burettes used for measuring samples or reagents results in cloudy solutions. A mixture of equal parts of glycerol and sucrose ground to a paste makes an excellent lubricant for this purpose.

Using the Evelyn colorimeter with a 420 mμ filter (Evelyn 1936) to measure the concentration of the yellow trimethylamine picrate, the range of linear response is rather limited. Galvanometer readings below about 45 per cent transmission indicate quite low recovery. This effect was not observed using the Klett-Summerson colorimeter with its blue filter. Incomplete extraction of the trimethylamine by the toluene at the higher trimethylamine concentrations was not responsible for the apparent low recovery when the Evelyn 420 mμ filter was used; re-extraction of the alkaline solution after the toluene from the first extraction had been removed showed that all the trimethylamine had been extracted in the first toluene extract, even in tubes containing 0.1 mg. trimethylamine nitrogen, using the regular procedure. Thus the Evelyn 420 filter must not be suitable for this particular colour.

The absorption curve of the trimethylamine picrate at two concentrations was measured with the Beckman Spectrophotometer (fig. 1). Two maxima were found, one at 410 mμ in the visible and one at 345 mμ in the ultraviolet. Comparison of this curve with the transmission curve of the Evelyn 420 filter showed the inadequacy of this filter. The transmission curve of the Evelyn 400 mμ filter, however, seemed to be satisfactory. Standard solutions of trimethylamine hydrochloride were made up and a set of picrate solutions made using the standard procedure (Dyer 1945). These were measured using the Evelyn 400 and 420

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filters, and the Beckman spectrophotometer set at 410 mμ using a band width of about 1 mμ. Results are shown in fig. 2. The yellow trimethylamine picrate in toluene yields a straight line relationship between concentration and log I_0/I at

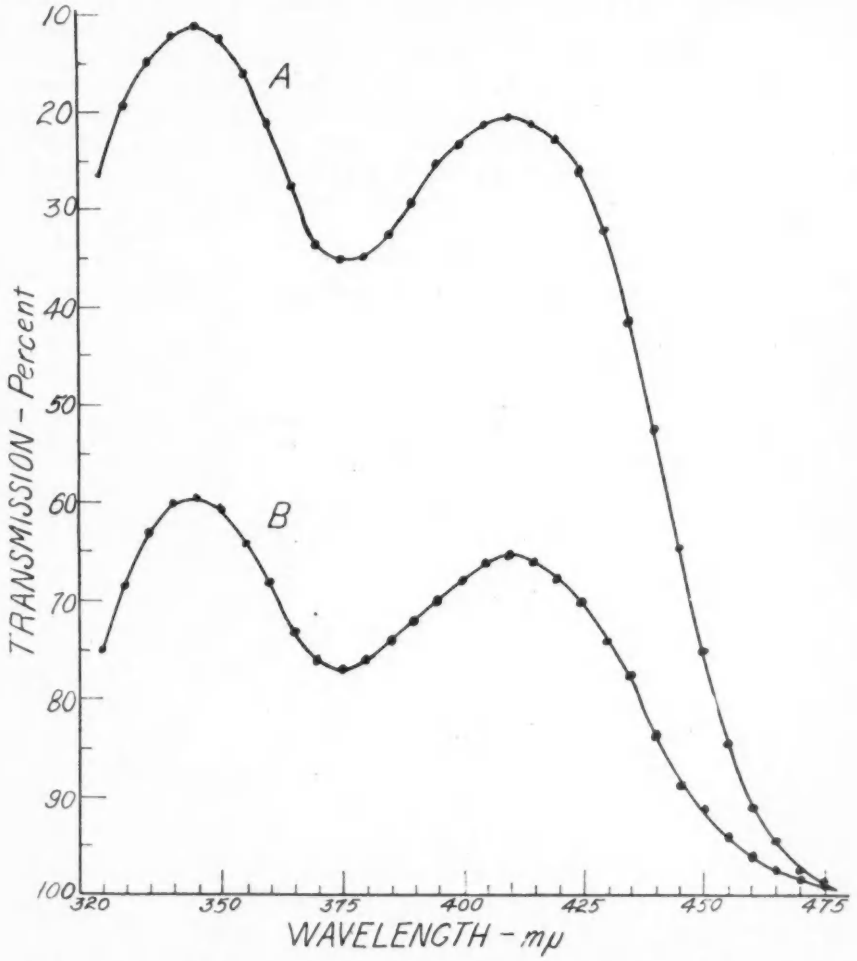

FIGURE 1. Absorption curves of trimethylamine picrate: A—extract from 0.036 mg. trimethylamine nitrogen; B—extract from 0.01 mg. trimethylamine nitrogen; measured with the Beckman spectrophotometer, slit width 0.1 mμ.

least down to a concentration of about 0.05 mg. trimethylamine nitrogen, galvanometer reading of about 10. Under the conditions used, the molar extinction coefficient, ϵ, is 5300. The Evelyn 420 filter, however, shows proportionality only

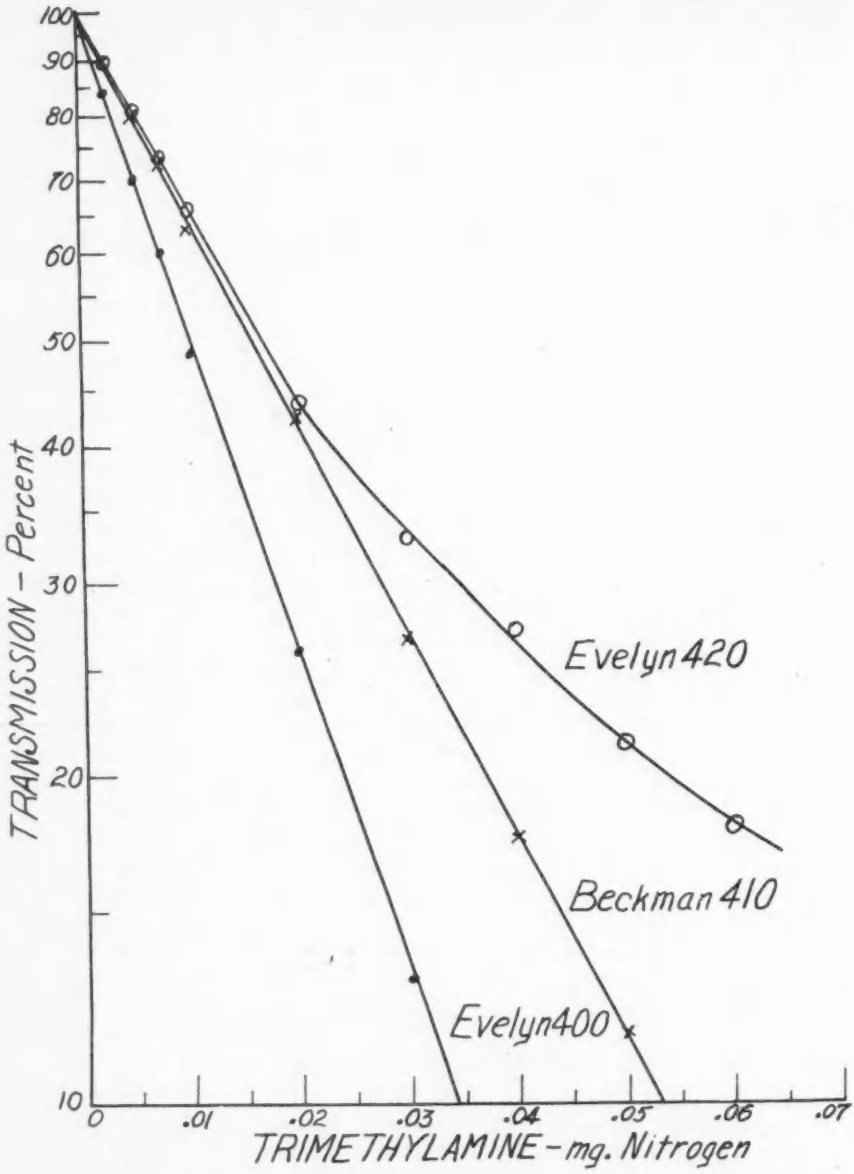

FIGURE 2. Calibration curves, transmission per cent (log scale) versus concentration of trimethylamine nitrogen—mg. used per tube, using the Evelyn colorimeter with 400 and 420 mμ filters and the Beckman spectrophotometer at 410 mμ.

down to a galvanometer reading of about 45, over which range the k value, 2-log galvanometer reading divided by the concentration of trimethylamine nitrogen in mg. per tube, is 18.0. The Evelyn 400 filter on the other hand gave a straight line down to a galvanometer reading of 10, k being 29.3. The sensitivity is also increased over the 420 filter about 60 per cent. The range of concentration which can now be measured satisfactorily is about 0.002 to about 0.035 mg. nitrogen in each tube, as compared with a maximum of about 0.02 mg. nitrogen with the 420 filter. An interference filter with a maximum transmission at 410 mμ with a band width of about 30 mμ was also tested but was not as good as the Evelyn 420 filter. The reason for this is unexplained.

The 400 filter has been found satisfactory in use.

ACKNOWLEDGEMENT

Acknowledgement is made here for the excellent suggestions given by Mr. D. G. Ellis.

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Amines in Fish Muscle. IV. Spoilage in Freshly Cut Cod Fillets

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ABSTRACT

The bacterial load on the surface of gutted cod is removed during filleting. Therefore the contamination of the fillet, and presumably its storage life, is determined by the sanitary conditions of the filleting operation. The storage period of the round fish has little relationship to the keeping quality of the fillet.

Spoilage of fillets from cod and similar fish has been shown to be a surface phenomenon (Wood, Sigurdsson and Dyer 1942). Dyer, Dyer and Snow (1946) have shown that in gutted fish the spoilage is similar. The focal area of bacterial growth was the skin surface and especially the exposed tissues lining the belly cavity, while the interior tissue remained free of bacteria until spoilage was well advanced. The actual spoilage of round fish was due to the products of bacterial action, principally volatile acids and trimethylamine formed from lactic acid and trimethylamine oxide in the flesh, which were formed at the surfaces and rapidly diffused into the interior tissue. Concomitantly, the lactic acid and trimethylamine oxide diffused out to the surface to replace that metabolized by the bacteria.

Since there is little or no bacterial contamination in the interior tissue, the surface of a fillet when cut should be sterile or contain few viable bacteria. Actually the bacterial load will be determined by the contamination during the filleting and packing operations. Thus the storage life of a fillet need have little relationship to the time the gutted fish is stored on ice previous to filleting.

EXPERIMENTAL

Fresh gutted cod were obtained from shore boats and stored in crushed ice before they were more than a few hours out of the water. They were obtained on successive days such that fish 1, 2, 3, 6, 8, 10 and 13 days on ice were available. These fish were filleted, washed, wrapped in waxed paper and stored at 3°C. Samples were removed daily for analysis, samples being taken from three fillets of each age on each day. The fillet samples were judged organoleptically and were analysed for trimethylamine content (Dyer 1945).

Initial Condition of the Fillets

Just before filleting, the gutted fish were graded by ten fresh-fish plant foremen.

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The fish 1 and 2 days old were graded as perfectly fresh, those 3 and 6 days on ice as good but not perfectly fresh, those stored 8 and 10 days as fair to borderline, and the 13 days old fish as spoiled by all the graders.

The average trimethylamine content of 3 separate fillets cut from each lot of these fish is shown in table I. Up to 6 days the trimethylamine had not accumulated beyond 1.4 mg. nitrogen per 100 g. Only the fish 13 days in ice showed an objectionable amount of trimethylamine. In the fish stored 8 and 10 days in ice, sufficient bacterial growth had taken place in the belly cavity and the skin to allow significant amounts of trimethylamine and other metabolic products to diffuse into the muscle later comprising the fillet.

TABLE I. Trimethylamine content of gutted fish stored 1 to 13 days on ice.

Age of fish days	Average trimethylamine content of 3 fillets, mg. N per 100 g.
1	0.8
2	0.8
3	0.9
6	1.4
8	3.8
10	3.0
13	14.0

The surface pH of the fillets from fish 1 to 10 days in ice varied between 6.3 and 7.1 with considerable variation between individual samples. Those 13 days in ice showed a higher pH value than the fresh fish, averaging 7 to 7.4.

SPOILAGE OF THE FILLETS STORED AT 3°C.

The average trimethylamine values of the fillets stored at 3°C are shown in fig. 1.

It is immediately evident from the graph that there is a lag period of 2 to 3 days before any trimethylamine increase occurs, regardless of whether the fish have been previously stored 1 or 13 days on ice. This is striking when it is realized that heretofore it has always been considered that there is a steady increase in spoilage products formed by bacteria starting from the time the fish was caught and continuing until it is consumed unless halted by freezing, cooking, or some other means. This point will be discussed later.

The trimethylamine curves for the fish 1, 2, 3 and 6 days on ice are almost identical, the increase starting at about 3 days, reaching a level of 15 mg. nitrogen per 100 g. tissue at a little over 4 days storage at 3°C and then rapidly increasing. The fillets from fish 8 and 10 days on ice start at a level of 3 or 4 mg. trimethylamine nitrogen per 100 g. tissue, then show a slight increase at 2 and 3 days, then increase in a manner parallel to the previously described group. They reach a

level of 15 only slightly sooner than the former at about 3½ days. The fillets from the fish 13 days in ice behave similarly; starting at a trimethylamine content of 13 mg. nitrogen per 100 g., it has of course already reached the spoilage threshold but even so an additional trimethylamine production equivalent to 15 mg. nitrogen per 100 g. is not produced until about 4 days.

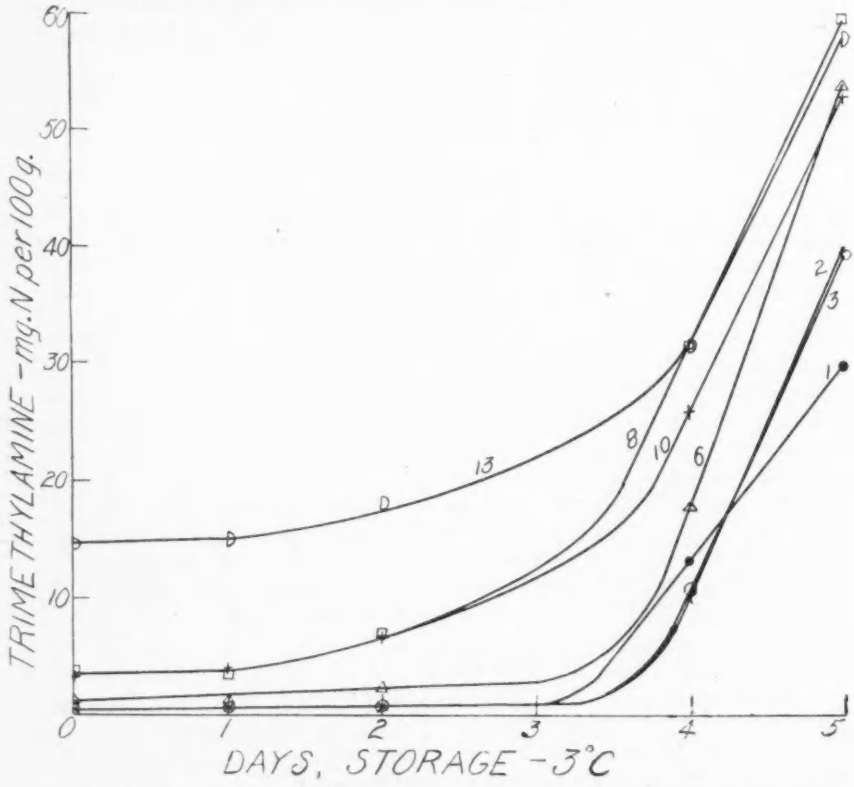

FIGURE 1. Trimethylamine rise in fillets cut from gutted fish stored 1, 2, 3, 6, 8, 10 and 13 days in crushed ice, stored at 3°C.

DISCUSSION

The results show that the length of storage of the gutted fish on ice does not affect significantly the subsequent keeping time of the fillets. The bacterial growth on the visceral cavity and skin surfaces is removed by the filleting operation and changed to a new locus on the surface of the newly cut fillet, which has been variously contaminated depending on the cleanliness of filleting. A lag period, about 3 days under the conditions used, is required before bacterial growth can again be initiated and significant amounts of spoilage products can accumu-

late. This interruption of bacterial growth affects the total keeping time of the fish, that is the time from catching and icing to the end of the storage life of the fillet. The time taken for the fillets described in the experiments above to reach a trimethylamine value of 15 is shown in table 2.

TABLE II. Total storage times required for iced fish and fillets to reach a trimethylamine value of 15.

Gutted fish on ice Days storage	Fillet, days storage at 3°C. to trimethylamine of 15	Total storage time days
1	4	5
2	4	6
3	4	7
6	4	10
8	3.5	11.5
10	3.5	13.5
13	1	14

From the standpoint of determining the maximum time that can elapse until the trimethylamine reaches 15, the usual organolpetic borderline for cod and similar fish (Dyer and Dyer 1949), the longer the period as gutted fish in ice the better. The accumulation of spoilage products takes place faster in the fillet than in the round fish. When it is considered that in both cases the spoilage is occurring on the surfaces and the metabolic products are diffusing into the flesh the reason becomes clear. In the thinner fillet the diffusion path is much shorter and the concentration can build up faster. This is the same reason that the napes in gutted fish spoil faster than the main body of the fish. But there is another factor concerned. Taste panel experiments (Dyer and Dyer 1949) have shown that the palatability of cod and haddock may be reduced during storage independently of bacterial action and hence the maximum storage life of these species may be determined by factors other than the bacterial growth, if optimum quality is to be maintained.

It seems obvious from the above results that a major factor controlling the keeping quality of the fillets is the degree of contamination during the filleting process. The bacterial load introduced at this point will control the length of the lag period and the rate of increase of spoilage products. This can only be reduced by thoroughly prewashing the gutted fish before filleting and keeping the filleting lines as clean as possible. Under laboratory conditions fillets have been cut out with so little contamination that they kept in an acceptable condition for 3 weeks or longer at 3°C (unpublished and Beatty and Gibbons 1937). Of course the fillets should be stored at temperatures as close to the freezing point as possible where bacterial growth is slowest (Hess 1932).

As shown in fig. 1, there is a slightly earlier trimethylamine rise in the fillets from the gutted fish stored longest on ice. One reason for this may be that

there was a slightly larger carryover of bacteria from the more heavily contaminated older fish onto the fillets. Another is that the types of bacteria are known to change as spoilage progresses (Dyer 1947, Wood 1940). Thus the proportion of different types of bacteria transferred to the fillets will vary and these will have different spoilage potentials (Castell and Anderson 1948). Thirdly, the leaching of soluble materials from the iced fish, particularly lactate and trimethylamine oxide, may change the suitability of the substrate for bacterial growth.

The fact that bacterial growth occurs on the surfaces of the fillet and the round fish also explains why surface pH is not a good measure of spoilage in freshly cut fillets. In the gutted fish stored in ice the small amount of alkaline trimethylamine diffusing in is not sufficient to change the pH of the heavily buffered muscle significantly. On the other hand, where the spoilage has occurred on the stored fillets there is a considerable accumulation of trimethylamine and other spoilage products on the surfaces (Dyer, Sigurdsson and Wood 1944, Elliot 1947) and the surface pH change becomes sufficiently large to use as an indication of spoilage.

SUMMARY

During filleting the locus of bacterial growth is changed from the visceral cavity and the skin of the gutted fish to the surface of the fillet. Thus the length of time the gutted fish is stored on ice before filleting has almost no influence on the keeping quality of the fillets cut from the fish.

The removal of the heavy bacterial load of the gutted fish during filleting with the consequent interruption of bacterial growth results in an increased total keeping time when the fish are filleted after a period of storage in ice over that of the keeping time of the iced fish or the fillets alone, especially if the fillets are stored at temperatures as near freezing as possible.

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Proteins in Fish Muscle I. Extraction of Protein Fractions in Fresh Fish

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ABSTRACT

Methods for the extraction of protein from fish muscle have been studied. Using the Waring Blendor to obtain fine subdivision, up to 95 per cent of the fish muscle protein can be extracted with 5 per cent sodium chloride. Optimum pH for extraction was pH 7-9, and the optimum salt concentration 3 to 5 per cent. About 3 per cent stroma protein, collagen and elastin, was found in cod and haddock muscle. Myosin constituted about 75 to 80 per cent of the total protein. Globulin X, myogen, and myoalbumin made up about 20 per cent of the protein.

Changes in the protein components make an important contribution to the deterioration of quality in stored fish. In this investigation, we have studied methods for the complete extraction of protein from the muscle cells and the fractionation of the extracted protein into its major components.

The extensive literature on protein chemistry includes few references to fish proteins. The muscle proteins of fish are considered to be very similar to mammalian proteins. The early classical works of Kuhne (1859), Haliburton (1888), and VonFurth (1918) are well-known. They developed the concept of a water soluble fraction, myogen—an albumen type protein—and a salt soluble fraction, myosin—a globulin. Historically, the work on muscle protein fractionation has usually been related to study of the mechanism of muscle contraction and the explanation of rigor mortis. The development of our present knowledge of muscle protein fractions has been well reviewed by Bailey (1944), and therefore reference will be made only to those papers pertinent to this investigation. Smith, in a series of papers starting in 1927, studied the muscle proteins in rabbit muscle. He investigated the solubility of the myosin fraction in salt solutions in relation to pH, protein concentration, and salt concentration (Smith 1934a). His work culminated in a scheme for the approximate separation and estimation of the muscle protein fractions (1934). He also studied the properties of the fractions obtained (1935, 1937).

Reay (1933, 1935) and Reay and Kuchel (1937) applied the methods of Smith to haddock muscle and found protein fractions similar to those present in rabbit muscle. About 85 per cent of the protein could be extracted with 7 per cent lithium chloride and about 95 to 97 per cent with successive extractions

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with 0.013 N. HCl. These workers found that the soluble protein was reduced on frozen storage. Finn (1932, 1934) studied the precipitation of protein in frozen press juice of ox and of halibut muscle and obtained about 20 per cent "denaturation" which was assumed to be due to changes in the myogen fraction, but which from subsequent work appears to be coagulation of myosin or globulin X.

The principal fractions present may be summed up as shown in table III. Myosin constitutes the largest fraction, about 65 to 75 per cent. Myogen constitutes about 10 per cent. Present in smaller amounts are globulin X and myoalbumin. These four constitute the intracellular proteins. The stroma proteins, collagen and elastin, together make up about 3 to 5 per cent of the total protein. In addition there is about 0.5 per cent of the protein present as nucleoproteins, hemoglobins, etc. It is realized, of course, that these fractions may not be pure proteins and some, at least, may contain carbohydrates and lipids. They can be separated further into various fractions often of doubtful significance, but Szent-Gyorgyi (1947, 1948) has recently made an outstanding contribution by fractionating myosin into two fractions, actin and "true myosin." These combine to form myosin as it has been commonly termed, and as it will be referred to in this paper.

EXPERIMENTAL

Determination of Protein

Total nitrogen in the fish muscle was estimated by the Kjeldahl-Gunning-Arnold method (A.O.A.C. 1945, p. 27) using 5 g. fish muscle. Nonprotein nitrogen was estimated by precipitation of the protein in 5 per cent trichloroacetic acid and determining nitrogen as above in an aliquot of the filtrate. A microkjeldahl method was used for determination of nitrogen in the extracts. Digestions were made in 30 ml. digestion flasks using the copper-selenium-phosphoric acid digestion mixture of Campbell and Hanna (1937). Distillation, about 5 mg. nitrogen per sample, was carried out in an automatic microstill (obtained from Scientific Glass Apparatus Co.), using N/28 HCl for ammonia absorption.

Extraction Methods

Various salt solutions have been used to extract the protein. Deuticke (1932) used a 10 per cent phosphate buffer of pH 7.2, Reay (1933) used 10 per cent NaCl, Smith (1934) tried various salts finding 7 per cent LiCl the best, and Greenstein and Edsall (1940) used 0.5 N KCl buffered with 0.03 M $NaHCO_3$. Smith's method consists of exhaustive extraction of muscle with solvent solution. 5 g. muscle are ground with 35 ml. solvent and 10 g. sand in a centrifuge tube. The mixture is shaken 30 minutes at 0°C, centrifuged and decanted. This grinding and shaking is repeated 6 to 9 times.

This procedure was tried on cod muscle using 7 per cent NaCl, 5 per cent NaCl, and 5 per cent LiCl, but only about 75 per cent extraction of the protein could be obtained. In addition, the method was laborious and time consuming. Froth formation and protein denaturation occurred. Finally, the Waring Blendor was tried but considerable denaturation occurred in the foam produced, resulting

in very low recovery of soluble protein. This was avoided by constructing a plastic plate which loosely fitted the blendor jar about 4 to 5 cm. below the top, and below the surface of the solution, provided with a handle for holding the plate in place during blending. This effectively prevented foam formation by eliminating the incorporation of air in the vortex of the swirling liquid. Good disintegration of the tissue was obtained. In this way, 90 to 95 per cent of the protein of fresh cod or haddock muscle could be brought into dispersion. The degree of extraction was ascertained by the amount of protein in solution after centrifuging the extract for 30 minutes at 2000 to 4000 r.p.m.

Various salts were tried as solvents, and it was found that all were efficacious in dispersing the muscle protein when the Blendor was used. Normal solutions of c.p. salts adjusted to pH 7- 7.5 were used for extraction. Muscle, 45 g., was blended 3 to 5 minutes with 900 ml. of salt solution at a temperature of 0°C. The extract was centrifuged 30 minutes at 4000 r.p.m. and total and nonprotein nitrogen determined on the extract. Results (table I) are expressed as per cent of total protein nitrogen in the cod muscle analysed.

TABLE I. Efficacy of various salts as protein extractants.

Salt	Soluble protein nitrogen —per cent of total protein
$BaCl_2$	84
$CaCl_2$	87
KBr	80
KCl	82
KI	91
K_2HPO_4	86
K_2SO_4	87
LiCl	86
$MgCl_2$	91
$MgSO_4$	83
Na acetate	77
Na_3 citrate	83
NaCl	91
$NaHCO_3$	77
Na_2SO_4	83

The variation is in the range of 77 to 91 per cent extraction. NaCl, $MgCl_2$, and KI all gave 91 per cent extraction and there seems to be no effect due to valency of ions or to a lyotropic series as was found by Gortner *et al.* (1928) with wheat proteins. It was concluded that the most important point in the extraction of the protein was sufficiently fine subdivision of the muscle fibrils, and that when this was obtained by use of the Blendor, the type of salt used for extraction was not critical.

Using 5 per cent NaCl with 0.02 M $NaHCO_3$ to give a pH of 7 to 7.5, it was found that the same percentage extraction was obtained when either 10, 30, or 50 g. cod muscle was extracted with 900 ml. of salt solution, 89, 89, and 90 per cent, respectively, of the total protein being extracted. Accordingly a 1 to 20 ratio of fish to extractant solution was adopted to give a high protein concentration.

The effect of temperature of extraction is quite marked. With fresh fish, extraction with 5 per cent NaCl at 5°C and at 25°C gave 91 and 92 per cent extraction of total protein. However, after being frozen and stored one week at − 18°C, the same fish was extracted at 5, 15 and 25°C; and 74, 65 and 60 per cent protein, respectively, was extracted. These differences were confirmed in subsequent work. Apparently, the stored fish had become sensitized to the higher extraction temperatures. Accordingly extraction is made at temperatures about 0°C by placing the salt solution in a cold room until ice crystals appear. Subsequent warming during blending then does not raise the temperature above 5°C.

Removal of Stroma and Unpeptized Protein

Attempts to filter the salt extract with the aid of filter paper pulp were unsuccessful, and it was found that centrifuging was the only practical means of separating the stroma and unpeptized protein from the solution. Centrifuging the extract at 2000 to 3000 r.p.m. for 30 minutes was found satisfactory, though occasionally about 1 per cent more protein was precipitated if longer times or faster speeds were used. Normally, the extracts could be stored at 0 to 3°C for 1 week either before or after centrifuging without affecting the soluble protein nitrogen or the myosin content.

The amount of residue varied between 3 and about 15 per cent depending on the condition of the fish. When about 90 per cent soluble protein was removed in the first extraction, re-extraction of the residue with 5 per cent salt yielded only about another 2 per cent of the protein nitrogen. The remainder, probably denatured myosin and possibly globulin X, could be removed by exhaustive extraction with N/100 NaOH leaving a residue of 3 to 4 per cent of stroma proteins.

Precipitation of Myosin

Smith (1934) found that a constant fraction of 88 per cent of the myosin could be precipitated by a 1 to 10 dilution of the salt extract with cold distilled water. He recommended storing the cold diluted extract for from 4 to 24 hours in the refrigerator. This was confirmed with fish protein in the present investigation. Before 4 hours the myosin gel was not sufficiently aged and did not precipitate quantitatively in the centrifuge. After 24 hours, the results were often erratic. Low results were obtained if the diluted extracts were stored at temperatures higher than 5°C. By dissolving the myosin obtained in 5 per cent NaCl and reprecipitating by dilution, it was found that 88 per cent of the fish myosin was precipitated by this procedure, in agreement with Smith's results on rabbit myosin. Szent-Gyorgyi (1947) has shown that it is extremely difficult to remove

potassium or sodium ions from myosin under these conditions, and this accounts for a fraction of the myosin remaining in solution.

Salt Concentration

The effect of the concentration of NaCl from 0 to saturation on the extraction of the protein was determined. Fresh cod muscle, 45 g., was extracted with 900 ml. of salt solution at 0°C using the Blendor. At salt concentrations above 2 per cent by weight, the pH of the salt solutions was buffered with 0.02 M $NaHCO_3$ to pH 7.2. At salt concentrations of 2 per cent and below no buffer was used, since at these low salt concentrations gels are obtained which cannot

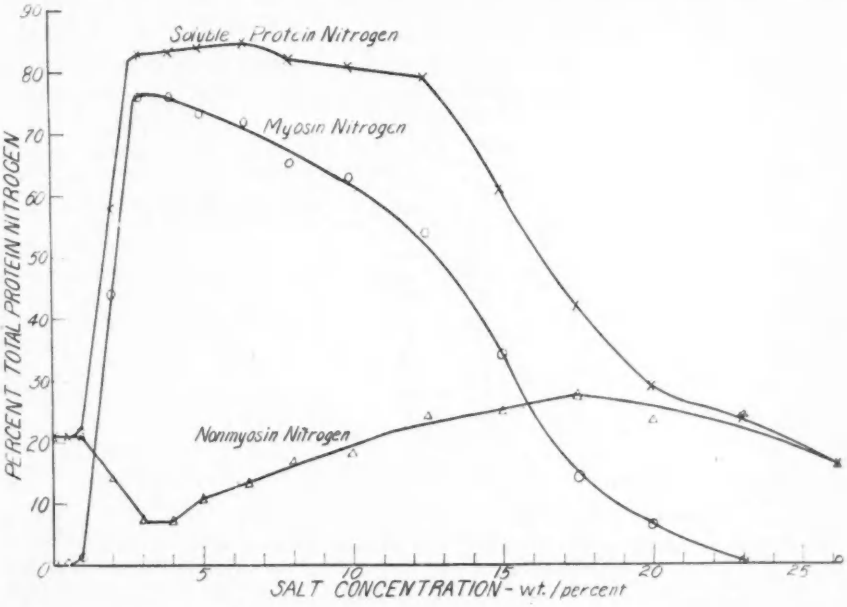


Figure 1. Effect of NaCl concentration on the extraction of soluble protein nitrogen, myosin nitrogen, and nonmyosin protein nitrogen from 45 g. cod muscle with 900 ml. salt solution at 0°C.

be centrifuged at a pH of about 7 or above, and the final pH after extraction in these cases was pH 6.6 to 6.7. The protein nitrogen content of the cod muscle was 2.50 per cent and the nonprotein nitrogen 0.40 per cent of the total weight of the muscle. The results are given in fig. 1, and are expressed as per cent of the total protein of the muscle. Myosin was determined by dilution to 0.5 per cent salt content with aging and centrifuging as usual. The results shown are corrected for 88 per cent precipitation.

Maximum extraction of soluble protein, about 85 per cent of the total protein, was obtained between 3 and 7 per cent salt. There is a very rapid increase

between 1 and 3 per cent salt which agrees with the results of Smith (1935) and of Szent-Gyorgyi (1948) on mammalian muscle. Myosin is precipitated in strong salt solutions, and it is seen from fig. 1 that there is a gradual decrease in the soluble protein at salt concentrations from about 12 per cent up to saturation. The myosin curve is similar with maximum extraction at 3 to 5 per cent salt, none being soluble at 1 per cent salt or in lower concentrations or again in saturated salt solution. The curve for nonmyosin nitrogen, about 10 to 20 per cent of the total protein, shows variations probably due to varying amounts of globulin X and myoalbumin being extracted along with the myogen.

pH of Extractant Solution

The effect of pH on the protein extraction using a 5 per cent NaCl solution was next investigated. 100 ml. of buffer solution adjusted to the desired pH with N/5 NaOH or N/5 HCl were added to 900 ml. of salt solution to give 5 per cent

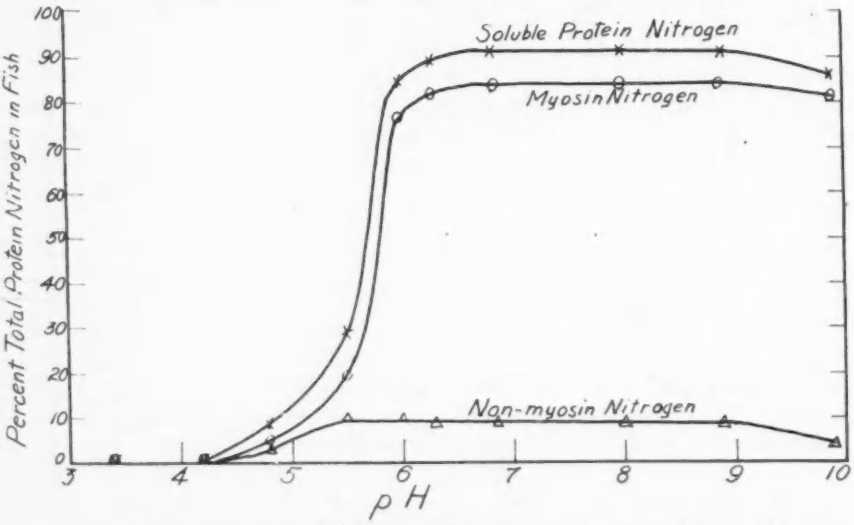


Figure 2. Effect of pH on the extraction of soluble protein nitrogen, myosin nitrogen, and nonmyosin protein nitrogen from 45 g. cod muscle by 900 ml. of 5 per cent NaCl solution.

salt concentration. 50 g. cod muscle was blended with the extractant as usual, and the final pH measured by the Beckman pH Meter. The buffers used and the pH of the solutions after extraction are shown in table II. The protein nitrogen of the cod muscle used was 2.38 per cent. Myosin was determined as usual with a 1 to 10 dilution in cold distilled water and the pH was adjusted to 7 after the dilution. The results, fig. 2 and table II, are corrected for 88 per cent myosin precipitation.

Maximum extraction of protein and of myosin is obtained between pH 7 and 9. There is a slight decrease between pH 6 and 7 and a very rapid decrease

from pH 6 to pH 5. Almost no protein is extracted below pH 5 in the presence of 5 per cent salt. It is known that these proteins are rapidly denatured in acid solution in the presence of salt, and in addition, the isoelectric point of the myosin is about pH 5.2, myogen 6.3, globulin X 5.2, and myoalbumin 3 (Weber 1934, Smith 1935, 1937). Thus these proteins would not be expected to be soluble at slightly acid reaction. No specific effect of the different buffers apart from pH could be noted.

TABLE II. Effect of pH on extraction of protein with 5 per cent NaCl

Buffer—Final Concentration	Final pH	Protein nitrogen extracted as per cent total protein in fish			
		Soluble Protein N	Myosin N	Nonmyosin N	Insoluble N
0.005 M KHPhthalate	3.4	0.4	0	0	100
"	4.2	0	0	0	100
0.05 M Acetic acid—Na Acetate	4.8	8	4.5	3.5	92
0.02 M " "	5.5	29	19	10	71
0.01 M Glycine	6.0	85	77	8	15
0.005 M KH_2PO_4	6.3	89	82	7	11
"	6.85	92	84	8	8
0.005 M H_3BO_3	6.85	91	83	8	9
"	8.0	92	84	8	8
"	8.9	92	84	8	8
"	9.9	86	82	4	14

MODIFIED PROCEDURE FOR EXTRACTION OF SOLUBLE PROTEIN AND MYOSIN

From the above results, a method was adopted for determining the approximate amounts of soluble protein nitrogen, myosin nitrogen, and insoluble nitrogen (stroma and denatured protein), which has been used in studies on the protein denaturation of fish in frozen storage.

Muscle tissue, 45 g., is blended 3 to 5 minutes with 900 ml. of extractant solution containing 5 per cent salt and about 0.02 M $NaHCO_3$ to give a pH of 7 to 7.5, and using the baffle plate described above to prevent foaming. The salt solution is stored slightly below 0°C to give a final temperature after blending of not over 5°C. The mixture is centrifuged 30 minutes at 2000 r.p.m. or faster. Soluble protein nitrogen is then determined on an aliquot of the supernatant solution, by the difference between total nitrogen and nonprotein nitrogen determined by microkjeldahl digestion and distillation or by a direct biuret method (Snow 1949). The insoluble nitrogen may be determined directly or from a determination of the total protein nitrogen of the muscle used.

Myosin is determined in another aliquot of the supernatant extract by adding 5 ml. to 45 ml. of distilled water at 0°C. After standing 4 to 24 hours in the refrigerator, the precipitated myosin gel is centrifuged off by spinning 30 minutes

at about 3000 r.p.m. After pouring off the supernatant, the myosin is dissolved in a few ml. of N NaOH, made up to volume and the nitrogen content determined either by the microkjeldahl or biuret method. Correction should be made for 88 per cent precipitation of myosin under these conditions.

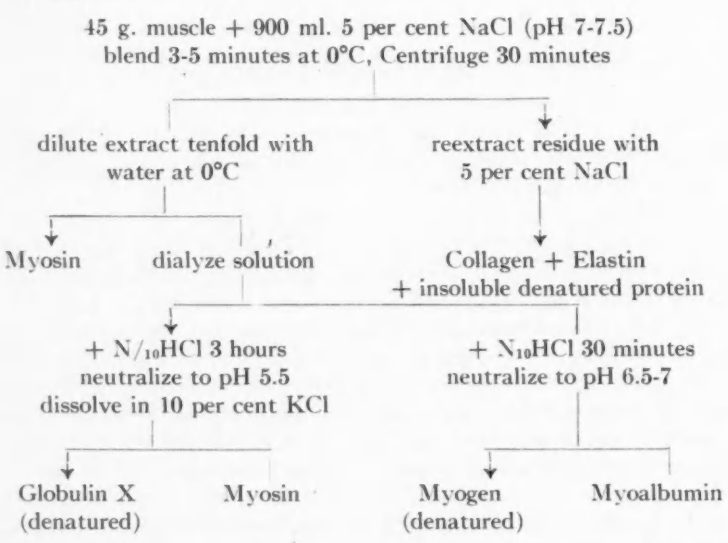
Fractionation of Cod Muscle Proteins

In table III is shown the fractions of muscle protein obtained by Smith (1937), Reay and Kuchel (1937), and Weber and Meyer (1933), and in addition the fractions found in cod muscle by the authors.

Table III. Protein fractions in muscle tissue

Fraction	Rabbit		Haddock	Cod
	Weber and Meyer	Smith	Reay and Kuchel	Dyer
Myosin	39	65	67	76
Globulin X	22	19	—	8
Myogen	22	10	18	6
Myoalbumin	—	1	—	7
Stroma	—	5	3	3

The method used in our work was very similar to that used by Smith (1937) which should be consulted for details. The modifications are obvious from the following diagram.



The results obtained in the separation of the fractions other than myosin were very variable and represent only the order of the concentrations present.

Further work is necessary to establish accurate values. Fish tissue seems to contain rather more myosin than rabbit muscle.

SUMMARY

Fish muscle proteins have been investigated and the results have shown that these are very similar to mammalian muscle proteins. Smith's method of salt extraction has been studied and very much shortened by the use of the Waring Blendor with a baffle to prevent foaming. The better subdivision of the muscle fibrils obtained allows a recovery of up to 95 per cent of the total protein present depending on the condition of the fish, and in addition it was found that neutral normal solutions of various salts were all efficacious in dissolving the protein when the Blendor was used, in contrast to the results of Smith. The temperature of extraction should be below 5°C. The optimum pH for solution of protein was pH 7 to 9 and optimum salt concentration 3 to 5 per cent sodium chloride. The amount of stroma proteins, collagen and elastin, was about 3 per cent in cod and haddock muscle, slightly less than the 5 per cent previously found in rabbit muscle.

Eighty-eight per cent of the myosin was precipitated from the salt extract by a 1 to 10 dilution with water. Myosin was the largest protein fraction present, about 70 to 80 per cent, slightly higher than in rabbit muscle. The other fractions, globulin X, myogen, and myoalbumin made up about 20 per cent of the protein present.

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Proteins in Fish Muscle. II. Colorimetric Estimation of Fish Muscle Protein

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ABSTRACT

A simple colorimetric method for protein estimation based on the biuret reaction has been developed. Protein concentrations of 0.1 to 1.2 mg. of nitrogen may be determined rapidly with an error of less than 2 per cent. Only one reagent is required.

A study of the rate of denaturation of fish muscle proteins (Snow 1949) required a large number of nitrogen determinations. A rapid method has been evolved that may have more general application.

Micro-Kjeldahl digestion and distillation procedures have been used with several modifications, but all are somewhat slow.

Various colorimetric tests, characteristic of either individual amino acids or linkages in the protein molecule, have been studied quantitatively. The Erlich reaction of triptophane (May and Rose 1922, Bates 1937) and the Folin phenol reaction of tyrosine (Folin and Denis 1912, Greenberg 1929), using the reagent of Folin and Ciocalteu (1927), have shown unsatisfactory precision when used with fish muscle extracts.

The Sakaguchi test of Albanese and Frankston (1945) as applied to protein estimation by Albanese *et al.* (1946) proved to be too cumbersome.

The biuret reaction was first introduced quantitatively by Riegler (1914) and subsequently presented in several modified forms. In most cases the precipitation of cupric hydroxide interferes (Fine 1935, Robinson and Hogden 1940), but Kingsley (1939) proposed a rapid simplified method for photoelectric colorimetry in which this precipitation was avoided.

On the basis of Kingsley's method a further modification has been developed, simplified by combining the cupric sulphate with the sodium hydroxide as a single reagent. An Evelyn photoelectric colorimeter was used to measure the colour.

Reagent

Sodium hydroxide, 180 g., and cupric sulphate, 2.0 g., are dissolved separately in about 400 ml. distilled water. The two solutions are mixed after the sodium hydroxide has cooled and diluted to one litre.

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The concentration of sodium hydroxide is not critical but the cupric sulphate must be controlled to this concentration in order to assure maximum colour formation over the widest range. The reagent will keep for two or three months and is not affected by light.

PROCEDURE

An aliquot of protein solution containing 0.10 to 1.2 mg. of protein nitrogen is added to a colorimeter tube and diluted to 5 ml. with distilled water. Five ml.

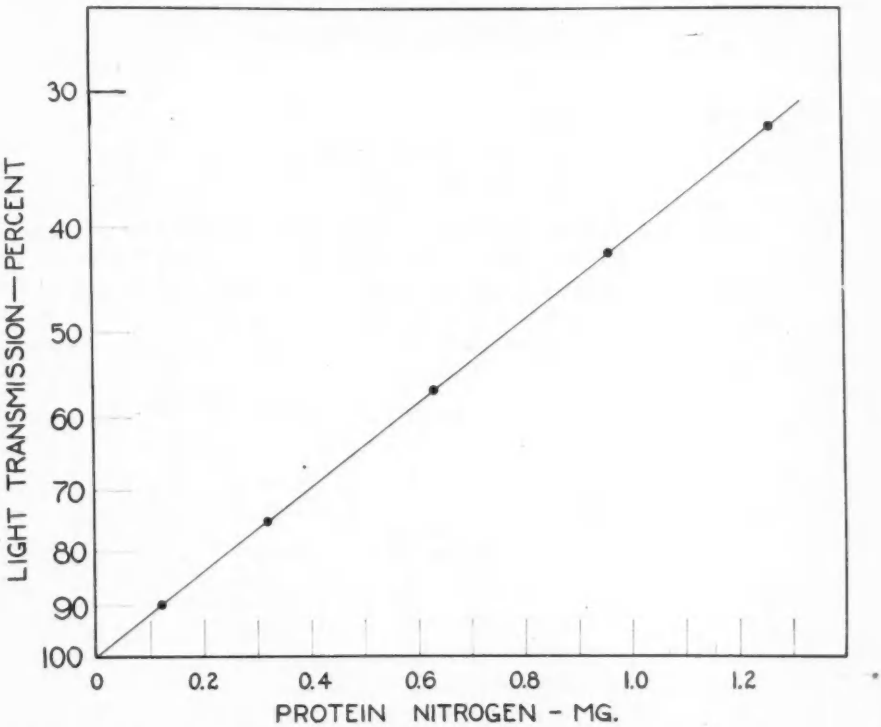

FIGURE 1. Standard curve for biuret colour. Per cent light transmission, logarithmic scale, plotted against protein concentration in milligrams of nitrogen.

of the biuret reagent are then added and the contents of the tube thoroughly mixed. Development of the violet colour is complete in 25 minutes but may become cloudy after 45 minutes. The colour intensity is read in the colorimeter with a filter having maximum transmission at 5400 Angstrom units, this wavelength having been determined with a Beckman quartz spectrophotometer. A blank tube is prepared simultaneously by adding 5 ml. of the biuret reagent to 5 ml. of distilled water.

This procedure is standardized by using various dilutions of a protein solution of known concentration, determined by a micro-Kjeldahl digestion and distillation developed in this laboratory and found satisfactory for this material (Dyer 1949). Percentage light transmission, logarithmic scale, is plotted against protein concentration expressed as milligrams of nitrogen to give a standard curve (fig. 1). The straight line relationship is in conformity with Beer's Law for the limits given. With the Evelyn colorimeter a K value may be used, this constant being equal to 2-log G divided by the number of milligrams of protein nitrogen present where G is the galvanometer reading when the blank is set at 100. A typical series of K-values for the globulin myosin have given an average of 0.390 ($\pm$ 0.004) using these units.

Albumin-Globulin Ratio

Since some workers have found a difference in the chromogenic values of serum protein fractions, there is a possibility of error in the determination of proteins in fish muscle extracts which contain both albumin and globulin fractions. The albumin of fish muscle was found to yield a violet colour of slightly greater intensity than the equivalent amount of muscle globulin. Table I gives the K values for these fractions and for mixtures composed of varying portions of each. Any inaccuracy from this source will be very slight if the standardization is carried out on a mixture having approximately the same ratio as the material analysed.

Table I. Albumin-Globulin Ratio

Fractions Present		K Value
Myosin (*per cent*)	Albumin (*per cent*)	
100	—	0.39
75	25	0.40
50	50	0.41
25	75	0.42
—	100	0.43

Interferences

A positive colour reaction with biuret reagent is given by polypeptides having three or more amino acid components and also by certain amino acids, viz., histidine, serine, and threonine. The presence of ammonia or ammonium salts permits the formation of deep blue cupric ammonium complex ion which becomes an interfering factor. Metallic ions forming insoluble hydroxides or sulphates with the reagent constitute another type of interference.

These substances are not normally present in a salt extract of muscle proteins. The non-protein nitrogenous constituents of fish muscle have been found to cause no interferences at the concentration in which they occur.

Accuracy of Results

Results obtained by the biuret procedure are compared with the micro-Kjeldahl values (table II) as an indication of accuracy. The colorimetric method affords a reasonably high degree of accuracy and precision. The difference between duplicate determinations is within the error due to individual variations in standard colorimeter tubes, giving results within 2 per cent of the micro-Kjeldahl values.

Table II. Accuracy and Precision

Sample	Protein (Mg. Nitrogen/ml.)			Error (*per cent*)
	Micro-Kjeldahl Method	Colorimetric Method		
1	0.975	0.980 0.960	Av. 0.970	− 0.5
2	0.625	0.636 0.626	0.631	+ 1.0
3	0.512	0.512 0.510	0.511	− 0.2
4	0.320	0.323 0.321	0.322	+ 0.6
5	0.270	0.267 0.270	0.268	− 0.6
6	0.153	0.156 0.153	0.155	+ 1.3

SUMMARY

A simple modified procedure using the biuret reaction has been developed for the estimation of fish muscle proteins. Only one reagent is required and no precipitation of cupric hydroxide occurs. The violet colour is measured with a photo-electric colorimeter and obeys Beer's Law for 0.1 to 1.2 mg. of protein nitrogen. Few interferences have been encountered and the method has proved to be accurate within 2 per cent. The procedure is primarily an adaptation for determining the concentration of fish muscle proteins in aqueous solvents but should be applicable to general protein work.

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Proteins in Fish Muscle. III. Denaturation of Myosin by Freezing

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ABSTRACT

The myosin fraction of fish muscle is isolated from the other proteins and is used in a study of denaturation by freezing as determined by solubility changes. The effects of the physical state of the protein, the rate of freezing, and the hydrogen ion concentration have been determined. Ions and salts showed a significant effect when the freezing temperature was lower than the cryohydric points of the salts.

Fractionation studies on muscle proteins have shown the existence of four distinct soluble fractions (Smith 1937). These include the salt-soluble fractions, myosin and globulin X, and the water-soluble fractions, myogen and myoalbumen. Recent work by Szent-Gyorgyi (1945) has shown still further division of the myosin fraction through dissociation, but, for the purposes of this study of fish muscle protein, the term myosin will be used in its broader application.

Protein denaturation, like protein structure, is a subject that is open to a considerable amount of controversy. The term is usually applied in an arbitrary manner and it has been used specifically in the frozen fish investigations to denote changes in solubility of the protein fractions.

Reay (1933) found that haddock muscle protein decreased in salt solubility after freezing and storage. Dyer (unpublished) showed that it is the myosin fraction which becomes denatured under these conditions.

The isolated myosin fraction has now been used for a more fundamental investigation of some of the factors influencing its denaturation by freezing.

METHODS

Muscle Protein Extraction

Smith (1934) obtained the soluble proteins from muscle by repeatedly grinding the material with sand and extracting it with lithium chloride solution. A method has been devised in this laboratory (Dyer *et al.* 1949) which requires only a single extraction in the Waring Blendor. In this procedure the muscle tissue is blended for 2 to 5 minutes with cold 5 per cent sodium chloride solution and the insoluble portion, namely, stroma proteins and denatured non-peptized protein, is centrifuged out. The extract contains the globulin, albumen and non-protein nitrogen fractions.

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Myosin Preparation

The myosin present in a salt extract can be precipitated by diluting to twenty times the volume with water (Smith 1934). The factors influencing this precipitation have been investigated thoroughly in the present study, to determine the limits for dilution, acidity and time. The degree of dilution of the 5 per cent salt extract should not be less than six times the volume of extract used. The effect of hydrogen ion concentration has been demonstrated, maximum precipitation occurring at pH 5 to pH 8 and dropping off rapidly beyond these limits. The time required for maximum precipitation at 5°C. has been found to be four hours, with prolonged standing causing no further change until the onset of spoilage.

The quantity of myosin precipitated under these conditions was 88 (± 2) per cent of the total myosin present. This is in agreement with the findings of Smith (1937) although no experimental evidence could be obtained to support his view that this 12 per cent dispersion was due to the presence of inorganic phosphate.

A procedure based on these limiting factors has been adapted for the preparation of quantities of myosin for use in experimental freezing. The neutral extract of soluble proteins in 5 per cent sodium chloride solution is diluted six to eight times with cold distilled water and centrifuged after standing four hours. The precipitate is then washed and recentrifuged, the myosin now being in the form of a hydrophilic gel containing about 98 per cent water. When sodium chloride is added to this material, to a concentration of 5 per cent, the globulin is dispersed to give a colloidal solution or sol. Concentrated solutions of myosin prepared in this manner contain about 1 milligram of protein nitrogen per millilitre.

Protein Estimation

The majority of the large number of protein estimations required in these studies were carried out by the rapid colorimetric method using the biuret reaction (Snow 1949). The procedure was developed especially for this work, and has proven very satisfactory.

Nitrogen determinations were made by a micro-Kjeldahl modification developed in this laboratory (Dyér *et al.* 1949) using the digestion mixture of Campbell and Hanna (1937). This method uses an all-glass, steam-jacketed distillation unit, and the mixed indicator suggested by Conway and Byrne (1933) for the back titration of the standard acid.

FREEZING OF MYOSIN

Physical State of the Protein

A preliminary study of the globulin myosin showed that freezing caused extensive denaturation when the protein was in the form of a gel, but not when it was dispersed in sodium chloride solution as a sol. This indicates that either the physical state of the protein or the concentration of salt may be a controlling factor in the extent of denaturation.

Table I summarizes the results obtained when various forms of myosin were rapidly frozen in plastic centrifuge tubes. Freezing appeared to be complete in less than one hour, and after 15 to 20 hours the tubes were thawed. The salt concentrations in the samples were adjusted to 5 per cent and the protein solubility was measured. The values show that only the precipitated protein is denatured and the extent of this change increases with the aggregation of the hydrated protein particles.

TABLE I. Denaturation of myosin frozen in different physical states

State of protein	NaCl present (*per cent*)	Percentage denaturation
Dispersed Sol		
—dilute	5-20	4
—concentrated	5-20	5
Suspended Gel		
—dilute	0.5	50-55
—concentrated	0	73
Centrifuged Gel	0.5	85-90
—washed	0	84-88
Salted-out Precipitate	25	80

RATE OF FREEZING

Practical studies of protein denaturation in frozen fillets have been carried out by Dyer, French and Snow (unpublished). It was found that the extent of denaturation depends on the rate of freezing, more rapid freezing causing less denaturation than slow freezing.

The effect of the rate of freezing has now been demonstrated with the isolated myosin. Quantities of myosin gel containing 5 milligrams of nitrogen were frozen in both the suspended and centrifuged forms in a series of tubes at controlled rates. Denaturation was determined by measuring the solubility in 5 per cent

TABLE II. Denaturation of myosin gel frozen at different rates

Freezing time	Percentage denaturation	
	Suspended gel	Centrifuged gel
10 min.	45	77
1-1½ hours	56	81
3-4 hours	61	86
8-10 hours	66	89
24-30 hours	75	93

sodium chloride after thawing all the tubes at an uniform rate following 18 to 24 hours storage at − 24°C. The results are given in table II and show that the rate of denaturation as measured by changes in solubility parallels the freezing time. At all rates of freezing, the centrifuged gel becomes more insoluble than the suspended gel, apparently due to the more closely aggregated state of the protein.

Effect of pH

Earlier work on muscle juice (Finn 1932, 1934) and other proteins (Lewis 1926) showed that alteration in hydrogen ion concentration influences the rate of denaturation. In the present study, two series of experimental freezing tests have been carried out to determine the effect of pH on the denaturation of myosin over a range of pH 4 to pH 9.

The first series of tubes contained the protein as a suspended gel prepared by diluting 5 ml. of a concentrated solution in 5 per cent salt to 50 ml. with water. These tubes, each containing about 5 milligrams of protein nitrogen, were frozen in 30 minutes and stored at − 24°C. for 20 hours. The salt concentration was adjusted to 5 per cent after thawing, and the protein solubility determined. The solubility of unfrozen suspended gel was measured over the same pH range as a control. The curve (fig. 1, A) shows the solubility of both native and denatured protein. The extent of denaturation of suspended myosin gel is constant over the entire range of pH values used, with the exception of a slight increase at the isoelectric point (pH 5.2, Smith 1937). Fig. 1, B shows that within a comparatively short range, pH 6.5 to pH 7.8, there is no significant denaturation. Marked denaturation becomes evident at values either on the acid or alkaline side of this range.

Effect of Ions and Salts

The results of this investigation have already shown that the solubility of myosin dispersed in neutral salt solution shows no initial decrease when frozen. However, if other salts are substituted for sodium chloride, if the range of concentration is wide, or if the solutions are kept frozen for a longer period of time, a definite denaturing effect may be observed.

The myosin for use in these experiments with ions and salts was twice precipitated and washed during its preparation. All salt solutions were at neutral pH, and the myosin was used in equivalent quantities of 4 to 5 milligrams of nitrogen in each freezing tube. Protein aliquots were rapidly frozen at sodium chloride concentrations ranging from 0.5 to 25 per cent. The tubes were thawed after 20 hours of frozen storage, and denaturation was determined by measuring the insoluble protein when the salt concentration was adjusted to 5 per cent. The same procedure was repeated with calcium, magnesium and ammonium chlorides, basing denaturation on solubility change in 5 per cent calcium chloride, 10 per cent magnesium chloride, and 5 per cent ammonium chloride respectively, these concentrations being approximately equivalent.

The denaturation curves are given in fig. 2 with the various chloride concentrations expressed as normalities for comparison. When the myosin is

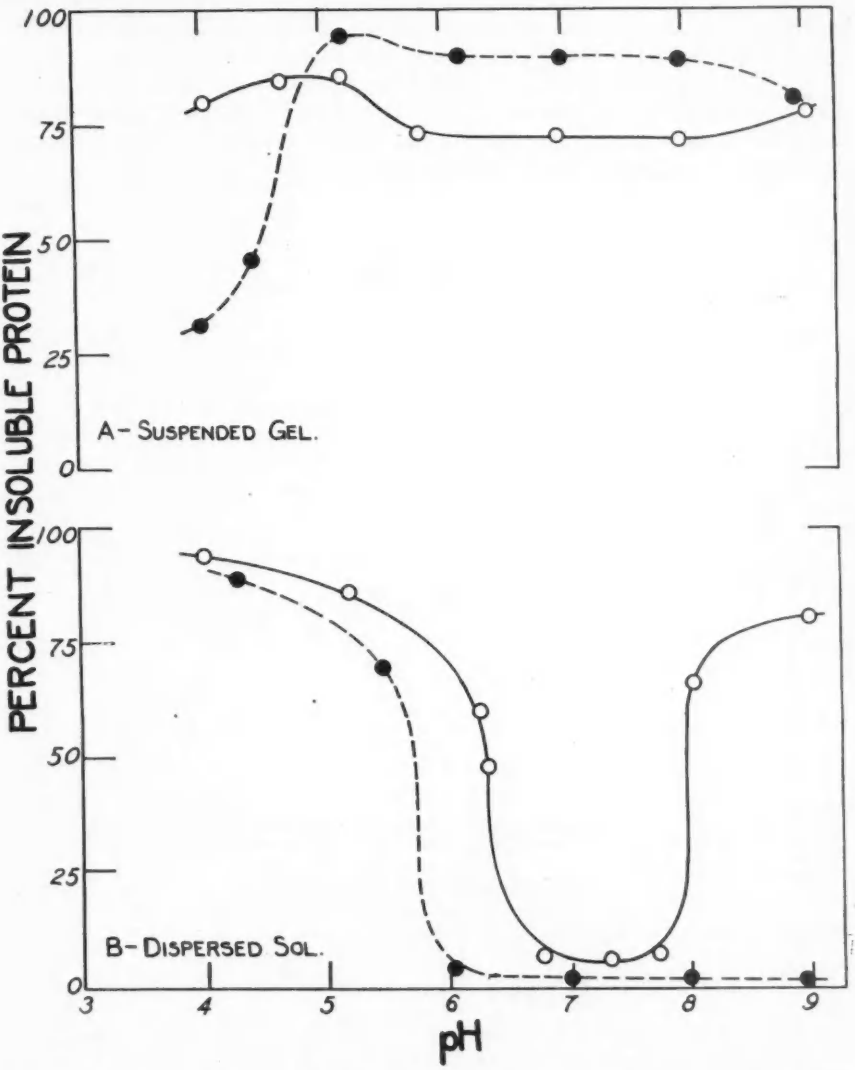

FIGURE 1. Effect of pH on denaturation of (A) suspended myosin gel (B) myosin dispersed in 5 per cent sodium chloride solution.

○———○ solubility in neutral 5 per cent sodium chloride after freezing at various pH values

●———● solubility of native protein at same pH before freezing

dispersed in solution there is no denaturation with any of the salts except ammonium chloride. At low salt concentrations the myosin is precipitated by dilution, and at high concentrations a salting-out occurs except with magnesium chloride. Under these conditions, where the protein is in the form of a precipitate, a corresponding degree of denaturation is seen. The behaviour of the sol in ammonium chloride solution is quite different from the other salts used, a large percentage of the protein being denatured at all of these concentrations.

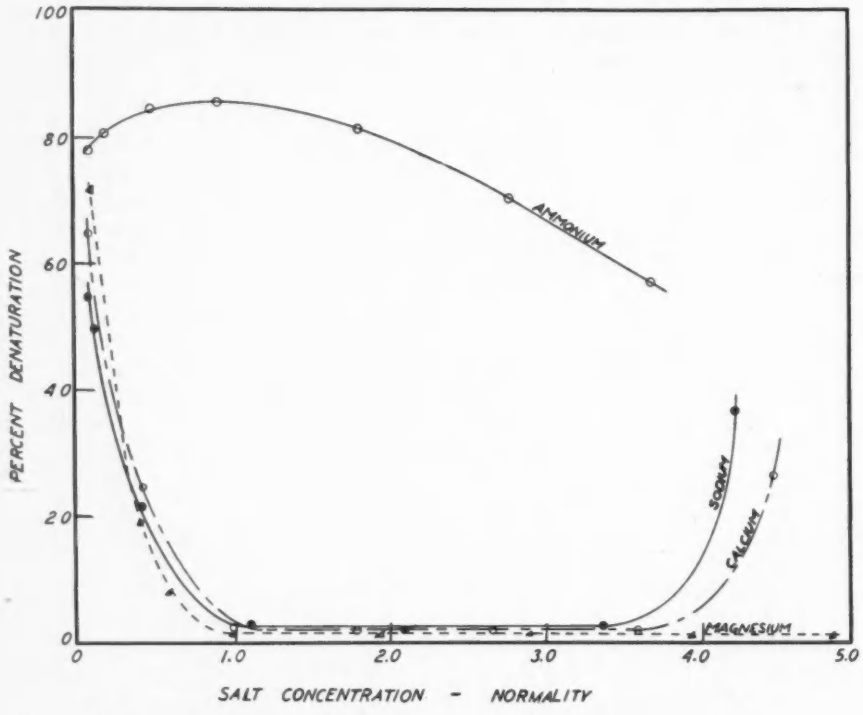

FIGURE 2. Denaturation curves of myosin frozen with different salt concentrations.
O———O ammonium chloride O———O calcium chloride
●———● sodium chloride Δ———Δ magnesium chloride

A second experiment was carried out in a similar manner to show the effect of storage time. Myosin was dispersed in 5 per cent solutions of sodium, calcium and ammonium chlorides, and in a 10 per cent solution of magnesium chloride. Aliquots of 20 and 40 ml. were frozen in tubes for 3 months at − 24°C. The results of periodic solubility analyses are shown in fig. 3. Only the protein dispersed in the ammonium chloride showed an immediate effect of the freezing. A progressively increasing effect was noted with sodium chloride after a few days but the calcium and magnesium chloride solutions were still unchanged at the end of three months.

At first the marked differences obtained with these chlorides appeared to be due to some specific ionic effect. Experiments with different anions also showed great variation in the extent of denaturation by freezing, but there was no apparent correlation with the effect of the cations. This suggests that some specific property of the salt itself is the governing factor in the rate and extent of denaturation rather than an ionic effect.

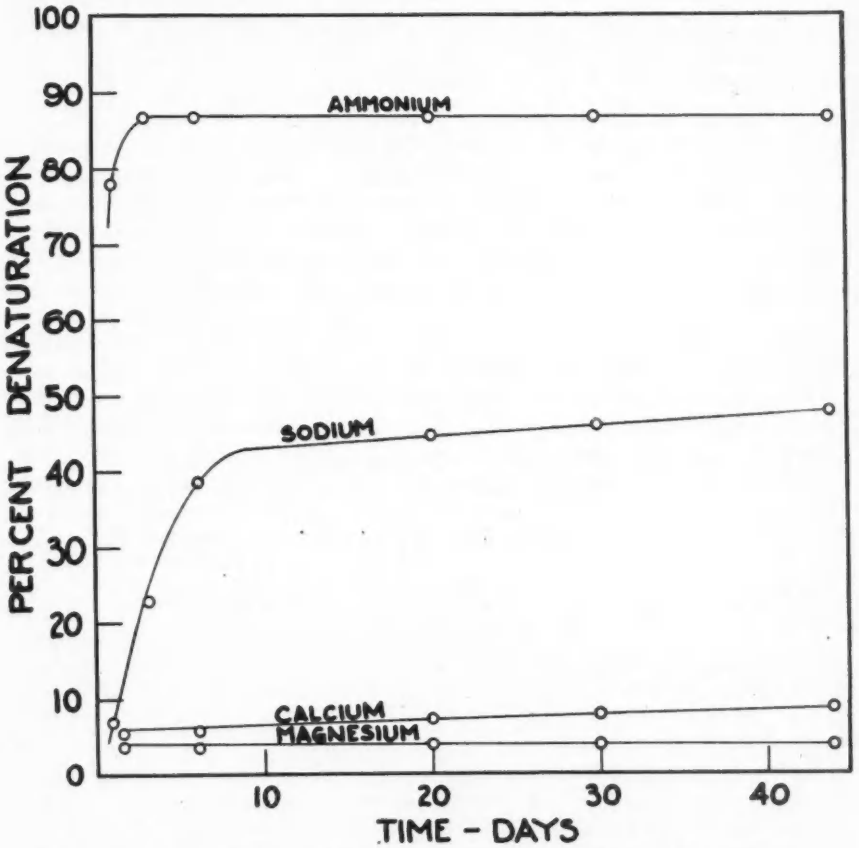

FIGURE 3. Effect of time of frozen storage on denaturation of myosin sol in normal chloride solution.

The significance of the eutectic temperatures or cryohydric points of the various salts has been seriously considered. The cryohydric point may be defined as the temperature level of a freezing solution at which the pure solid states of both components separate out together occurring at a specific concentration of solute. Therefore, true freezing would not be complete at a higher temperature, that is, an unfrozen aqueous phase would still remain. The above results indicate

TABLE III Significance of cryohydric point on denaturation of myosin sol by normal solution of salts

Salt	Cryohydric point (°C.)	Per cent denaturation at − 24°C.		
		1 day	7 days	90 days
Calcium Chloride	− 55	4	4	6
Magnesium Chloride	− 33.6	2	2	3
Sodium Chloride	− 21.1	4	40	51
Ammonium Chloride	− 15.6	87	88	88
Sodium Sulphate	− 1.0	86	86	87

that denaturation takes place only if the freezing temperature is lower than the cryohydric point of the salt used to disperse the protein.

The freezing data from the preceding experiment have been analysed in this respect in table III. Calcium and magnesium chlorides have cryohydric points much lower than the freezing temperature of − 24°C. and no denaturation occurs over a long period of time. The initial denaturation was quite extensive with ammonium chloride and sodium sulphate which were frozen well below the cryohydric values for these salts. Sodium chloride, frozen slightly below its cryohydric point, showed definite denaturation of the myosin after a few days.

Further evidence in support of this explanation was obtained by freezing tubes of myosin sol using a wide variety of salts as dispersing agents. The accumulated data are presented in table IV. The results with every one of the

TABLE IV. Denaturation of myosin sol frozen at different temperatures

Salt	Cryohydric point (°C.)	Percentage denaturation			
		− 4°C.	− 8°C.	− 24°C.	− 33°C.
Sodium Sulphate	− 1.0	36	95	86	80
Disodium Phosphate	—	45	95	80	67
Potassium Sulphate	− 1.2	83	73	84	71
Potassium Nitrate	− 2.9	*	78	79	73
Magnesium Sulphate	− 3.9	*	70	59	55
Barium Chloride	− 7.8	*	0	60	45
Potassium Chloride	− 11.1	*	0	78	44
Ammonium Chloride	− 15.8	*	0	88	50
Sodium Nitrate	− 18.5	*	5†	50	35
Sodium Chloride	− 21.1	*	4†	40	18
Magnesium Nitrate	− 29	*	3†	5†	16
Magnesium Chloride	− 33.6	*	*	0	0
Calcium Chloride	− 55	*	*	2†	0

*Normal solutions not frozen at this temperature
†Denaturation considered negligible

salts used are in agreement with the proposed explanation of denaturation effects. No appreciable denaturation occurred at temperatures above, but was quite marked below, the cryohydric point. The wide variations in the absolute values for percentage denaturation are due to the different freezing rates. These rates are much slower at the lower temperatures as well as with the salts having the lower cryohydric points. The extent to which the cryohydric point is lowered by the protein is not known, but it is believed to be slight at the concentrations in which these salt solutions were used.

SUMMARY

The globulin myosin is precipitated from a sodium chloride extract of fish muscle proteins by dilution. The isolated fraction is used for the study of some of the factors influencing the extent to which myosin is denatured when frozen, loss of solubility in 5 per cent salt solution being the criterion of denaturation.

The initial decrease in solubility obtained on freezing was found to depend on the physical state of the protein, a centrifuged gel showing almost complete denaturation whereas a sol showed no immediate change.

Rapid freezing causes much less denaturation than slow freezing.

A 5 per cent salt solution of myosin shows almost no denaturation over a narrow pH range, from 6.5 to 7.8, but rapid change in more acid or alkaline solutions.

Myosin solutions containing different ions have been found to be denatured to widely differing extents. This appears to be associated with the temperature of freezing and storage as related to the cryohydric point of the salt present. In solutions of all the salts tested, the myosin was denatured only when the temperature levels fell below the cryohydric points of the respective salts.

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The Acid-Soluble Phosphorous Compounds of Fish Skeletal Muscle

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ABSTRACT

Total phosphorus, inorganic phosphorus and phosphocreatine were determined in trichloracetic acid extracts of fish skeletal muscle, and the acid extracts were then fractionated for the determination of adenosine polyphosphates, fructose mono and diphosphates, glucose monophosphates, myoadenylic acid, pentose phosphate and coenzyme, while glycogen was determined in the extracted tissue. A comparison of the distribution of these compounds in fish and rat skeletal muscle was made.

Though the function and distribution of acid-soluble phosphorus compounds of mammalian muscle has been extensively investigated, especially during the past decade (Baldwin 1948, pages 339-342), there appears to be little published information regarding their occurrence in fish muscle. The fact that the myosin content of fish muscle (Reay and Kuchel 1937) is similar to that of mammalian muscle (Smith 1937), and that myosin and the enzyme adenosinetriphosphatase have not been separated, would suggest the presence of adenosinepolyphosphates in fish muscle. This suggestion is supported by the fact that pentoses are known to occur in this tissue (Berkeley 1921). Early attempts to demonstrate phosphocreatine (phosphagen) in fish muscle indicated either that the quantity present was rather small (Eggleton and Eggleton 1928), or that it was absent (Irving and Wells 1928). Hunter (1929) has pointed out that fish muscle contains creatine in amounts usually in excess of those found in mammalian skeletal muscle. The present experiments have demonstrated that the acid-soluble phosphorus compounds referred to above, together with a number of other phosphorylated muscle intermediates, occur in fish skeletal muscle.

EXPERIMENTAL

Fish weighing from about 50 to 200 g. were taken by dip net from the tanks of a local salt water aquarium during the winter months. During capture a certain amount of struggling occurred before the tissues were frozen, though every attempt was made to reduce this time interval to a minimum. The fish were either mechanically stunned and pieces of excised muscle dropped immediately into liquid nitrogen, or they were immersed whole in this freezing medium while

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still alive. The frozen excised muscle samples were powdered in a mortar in a room at about − 25°C with frequent additions of liquid nitrogen. It was found that in dissecting out muscle from whole small frozen fish small pieces of bone were accidentally broken off and included, though no similar difficulty was experienced with the unfrozen lingcod and tomcod samples. The powdered muscle samples were stored in closed glass containers at − 25°C and were analysed at convenient intervals. The following samples of frozen muscle powders were studied:

Leg muscles of a 175-g. white male rat. The muscle powder was used within one day of preparation.

Excised muscles of stunned starry flounder (*Platichtys stellatus*), blue sea-perch (*Taeniotoca lateralis*) and whiting (*Theragra chalcogramma*). The muscle powders were used after storing 37, 84 and 106 days respectively.

Excised muscle of a lingcod (*Ophiodon elongatus*) in *rigor mortis*. The muscle powder was used within one day of preparation.

A whole live whiting was immersed in liquid nitrogen and the muscle powder obtained from it was used within one day of preparation.

A whole live tomcod (*Microgadus proximus*) was immersed in liquid nitrogen. The frozen muscle from one half the fish was powdered and analysed within one day of preparation. The remainder was thawed and kept 2 days at 0°C when the muscle was frozen, powdered and analysed promptly.

The powdered muscle samples were extracted with successive portions of trichloracetic acid, and the extracts thus obtained were fractionated to yield a fraction containing barium compounds insoluble in aqueous solution at pH 8.2 (the "barium insoluble" fraction) and a second fraction containing barium compounds insoluble in aqueous 80 per cent ethyl alcohol at the same pH value (the "barium soluble" fraction), the solutions being kept cold (0-3°) throughout. The extraction and fractionation procedures and methods of analyses of the two fractions were those which have been fully described by Le Page and Umbreit (Umbreit, Burris and Stauffer 1948, pages 159-174). Certain minor difficulties were encountered in application of this technique to the samples being studied. Thus the high inorganic phosphorus content of the "barium insoluble" fraction made accurate determination of the phosphorus hydrolysed in 7 minutes at 100° C in 1 N HCl (Δ 7P) unreliable with most fish samples. For this reason no attempt was made to distinguish between adenosinediphosphate (A.D.P.) and adenosinetriphosphate (A.T.P.), calculation for these usually being made from the pentose content of the fraction and expressed as A.T.P. It was also found that the precipitate which was formed by ethyl alcohol precipitation was bulky and gelatinous, and that consequently small amounts of alcohol were usually carried into the "barium soluble" fraction and interfered during the attempted determination of phosphopyruvic acid by the alkali-iodine method. In view of this difficulty, and also because normal rat muscle contains no appreciable phosphopyruvic acid, the determination of this intermediate was not made.

The results of the analyses are recorded in table I. The percentage of trichloracetic acid extracted phosphorus which was recovered in the two fractions

TABLE I. Acid-soluble phosphorus compounds and glycogen of fish muscle compared with those of the white rat.

(Micromoles per 100 g.)

	*Rat (Le Page 1946a and b)	Rat (Present work)	Starry flounder	Whiting (1)	Whiting (2)	Tomcod	Tomcod (Muscle stored 2 days at 0°C)	Blue perch	Lingcod
Total P	5,500	7,170	12,400	8,970	15,300	11,300	5,790	8,250	5,670
Inorganic P	800	2,920	9,150	5,620	14,100	9,150	5,500	4,580	4,250
Phosphocreatine	1,600	1,470	1,680	845	45	0	0	553	535
A.T.P. plus A.D.P.	800	405	123	371	240	197	27	372	52
Fructose-1-6-diphosphate	25	29	13	128	27	35	20	45	4.7
Phosphoglyceric acids	400	618	88	352	119	103	52	85	35
Glucose-1-phosphate	100	87	39	56	42	58	49	29	120
Glucose-6-phosphate	400	204	47	97	9	38	39	136	58
Fructose-6-phosphate	10	44	6.8	82	23	8	17	25	6.5
Pentose phosphate	50	20	7.6	183	0	14	22	149	161
Myoadenylic acid	75	14	51	214	158	240	0	90	80
Coenzyme	25	16	3.8	11	5.7	5.0	2.4	5.0	8.5
Glycogen (as glucose)	2,000	2,720	127	630	140	156	39	267	167
P. recovered in the two fractions (%)	93-96	91	96	93	96	96	87	97	97

*These values were taken from histograms and thus are only approximate.

was usually within, or only slightly outside of, the range recorded for normal rat muscle by Le Page (1946a). It is assumed that the small amount of phosphorus not accounted for in these two fractions was included in the third "barium soluble alcohol soluble" fraction which was discarded. The compounds determined in the "barium insoluble" fractions accounted for 91 to 106 per cent, and those in the "barium soluble" fraction 87 to 99 per cent, of the total phosphorus of the respective fractions. Similar recoveries found for rat muscle by Le Page (1946a) were 95 to 103 per cent and 85 to 90 per cent respectively.

The distribution of phosphorus compounds in the sample of rat muscle analysed was in general similar to that found by Le Page (1946b) and by Conway and Hingerty (1946) for similar tissue. The rather high inorganic phosphorus, and consequently total phosphorus, values obtained with the rat muscle and fish muscle which was excised while frozen were probably accounted for by accidental contamination with bone splinters. The total phosphorus obtained with fish muscle which was excised while not frozen and was not contaminated with bone (lingcod and tomcod stored at 0°C) was closely similar to that of rat muscle. The phosphocreatine values were extremely variable, and only with one sample was a value similar to that found in normal rats obtained. It is suggested that the phosphocreatine of fish muscle is rapidly broken down when the fish struggles, and that this reaction is perhaps favoured by a rather high phosphokinase ("Lohmann enzyme") content in the tissues. This suggestion is supported by the fact that, as previously stated, other workers have never been able to demonstrate amounts of phosphocreatine in fish muscle which would correspond to its known creatine content.

The results show that the phosphorylated intermediates which occur in fish muscle are similar to those which are found in rat muscle. The quantities recovered were quite variable and usually lower than for rat muscle, but the recorded differences may have been due to variations in the amount of struggling which occurred before the fish tissues were frozen. The glycogen values were low, but this may have been due to a poor nutritional condition of the fish, or to the breakdown of glycogen before the samples were frozen. The muscle glycogen content of well nourished fish is usually quite high (400 to 600 mg. per cent), but it decreases rapidly when the fish struggle or when dead fish are stored in ice (Leim, Macleod and Simpson 1927, Macleod and Simpson 1927, Sharp 1934).

Little or no A.T.P. and myoadenylic acid were found in tomcod muscle which had been stored 2 days at 0°C, and the glycogen content was considerably lower than that found in the original tissue. On the other hand significant amounts of glucosephosphates and of the fructosephosphates remained. This may account for the fact that Macpherson (1932) found no appreciable change in "reducing sugar" in haddock muscle during 24 hours' storage in ice. Significant amounts of both these hexosephosphates occurred in the lingcod muscle.

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